DELINEATION OF SIGNALLING PATHWAY IN ALPHA ADRENOCEPTOR MEDIATED VASORELAXATION USING GOAT ARTERIAL STRIPS

A DISSERTATION SUBMITTED TO THE TAMIL NADU

DR.M.G.R.MEDICAL UNIVERSITY, IN PARTIAL FULFILMENT OF

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Department of Physiology

Christian Medical College, Vellore

May 2019

Department of Physiology
Christian Medical College
Thorapadi post,
Vellore 632002.
Tamil Nadu, S. India.

Phone: +91 416 228 4268
Fax: +91 416 226 2788, 226 2268
Email: physio@cmcvellore.ac.in

CERTIFICATE

This is to certify that the thesis entitled "**Delineation of signalling pathway in alpha adrenoceptor mediated vasorelaxation using goat arterial strips**" is a bonafide original work carried out by Dr. Alen Major Venis, in partial fulfillment of the rules and regulations for the M.D. – Branch V Physiology examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in May 2019.

Dr. Sathya Subramani
Professor and Guide

Department of Physiology
Christian Medical College
Vellore 632002

Department of Physiology
Christian Medical College
Thorapadi post,
Vellore 632002.
Tamil Nadu, S. India.

Phone: +91 416 228 4268
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Dr. Elizabeth Tharion
Professor and Head of Department

Department of Physiology
Christian Medical College
Vellore 632002

Department of Physiology
Christian Medical College
Thorapadi post,
Vellore 632002.
Tamil Nadu, S. India.

Phone: +91 416 228 4268
Fax: +91 416 226 2788, 226 2268
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Dr. Anna B. Pulimood
Principal

Professor, Department of Pathology
Christian Medical College
Vellore 632004

DECLARATION

I, Dr. Alen Major Venis, hereby declare that the investigations that form the subject matter for the thesis entitled "**Delineation of signalling pathway in alpha adrenoceptor mediated vasorelaxation using goat arterial strips**" was carried out by me during my term as a Postgraduate student in the Department of Physiology, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

Dr. Alen Major Venis
Postgraduate student

Department of Physiology
Christian Medical College
Vellore 632 002

PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled **"Delineation of signalling pathway in alpha adrenoceptor mediated vasorelaxation using goat arterial strips"** of the candidate **Dr. Alen Major Venis** with registration number **201615351** for the award of **M.D. Physiology (Branch V)** degree examination of **The Tamil Nadu Dr.M.G.R. Medical University, Chennai** to be held in **May, 2019**. I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains pages from the introduction to conclusion and the result shows **10%** of plagiarism in the dissertation.

Dr. Sathya Subramani
Professor and Guide

Department of Physiology
Christian Medical College
Vellore 632 002

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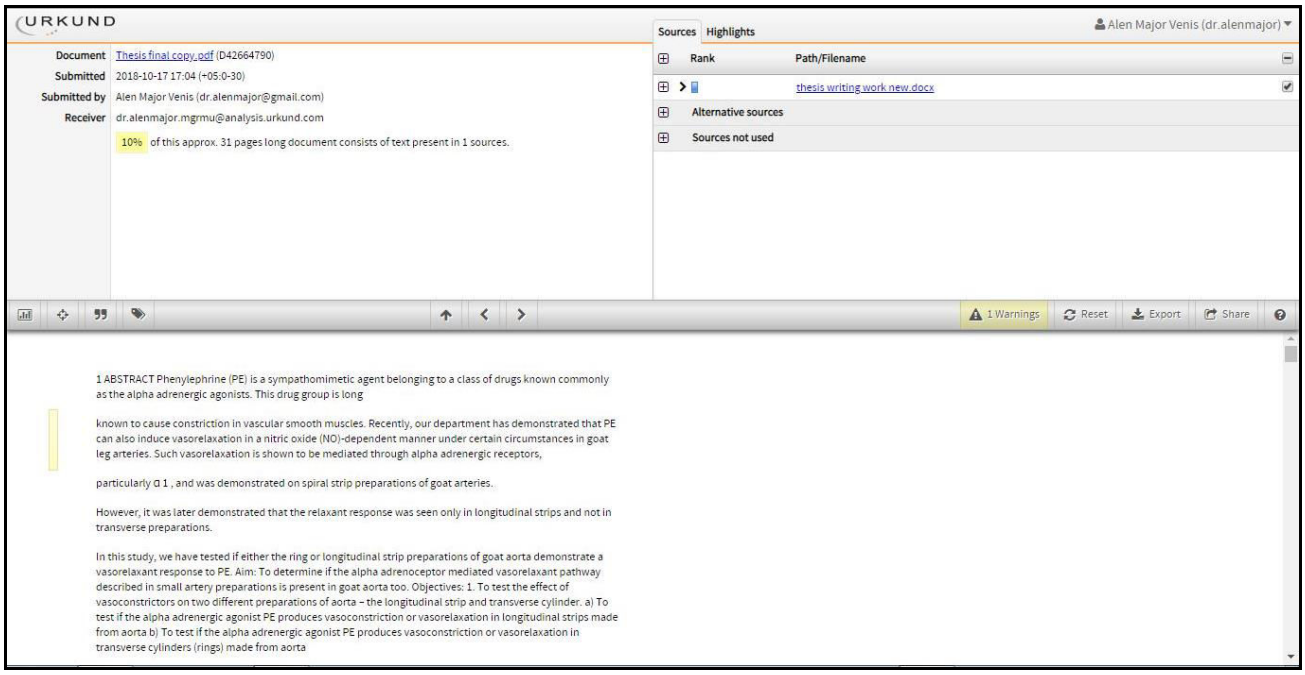

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CONTENTS

ABSTRACT

ABSTRACT

Phenylephrine (PE) is a sympathomimetic agent belonging to a class of drugs known commonly as the alpha adrenergic agonists. This drug group is long known to cause constriction in vascular smooth muscles. Recently, our department has demonstrated that PE can also induce vasorelaxation in a nitric oxide (NO)-dependent manner under certain circumstances in goat leg arteries. Such vasorelaxation is shown to be mediated through alpha adrenergic receptors, particularly α_1, and was demonstrated on spiral strip preparations of goat arteries. However, it was later demonstrated that the relaxant response was seen only in longitudinal strips and not in transverse preparations. In this study, we have tested if either the ring or longitudinal strip preparations of goat aorta demonstrate a vasorelaxant response to PE.

Aim:

To determine if the alpha adrenoceptor mediated vasorelaxant pathway described in small artery preparations is present in goat aorta too.

Objectives:

1. To test the effect of vasoconstrictors on two different preparations of aorta – the longitudinal strip and transverse cylinder.
    a) To test if the alpha adrenergic agonist PE produces vasoconstriction or vasorelaxation in longitudinal strips made from aorta
    b) To test if the alpha adrenergic agonist PE produces vasoconstriction or vasorelaxation in transverse cylinders (rings) made from aorta

2. If vasoconstriction occurs in either of the above cases, then to test the response of PE in the presence of NO donors, SNP and L-Arginine.
3. If vasorelaxation occurs in objectives 1a and 1b, or 2, to test if the vasorelaxation produced by PE alone or PE/NO combination is preventable by prazosin (specific α_1-blocker).

Methods:

Aortae were isolated from fresh goat hearts and were cut into rings or longitudinal strips. The preparations were then suspended in an organ bath of 25 ml capacity which was filled with physiological salt solution, maintained at 37ºC by means of a circulating water bath, and also aerated with carbogen (95% oxygen and 5% carbondioxide). One end of the aortic preparation was fixed to the organ bath and the other end of the isolated tissue was connected to a force transducer and tension was recorded using a data acquisition system (PowerLab from AD Instruments). Drugs were then added to the organ bath and any change in tension recorded by the force transducer was recorded. Data of the viable tissue was analyzed using SPSS 23.0 and visualized using Igor pro.

Results:

In both ring and longitudinal strips of aorta, Phenylephrine (PE) caused vasoconstriction under normal and high NO environment, unlike in small arteries.

Conclusion:

There is no alpha-adrenoceptor mediated vasorelaxant pathway in aortic smooth muscle.

INTRODUCTION

INTRODUCTION

Arteries are the major blood vessels carrying blood from the heart to different parts of the body. Based on their site and histology, arteries are classified into three main types: (a) Large sized elastic arteries, (b) Medium sized muscular arteries and (c) Small sized arterioles. The predominant innervation of vascular smooth muscle is the sympathetic nervous system by the release of the neurotransmitter noradrenaline, which is a non-specific agonist of adrenergic receptors present in them. The alpha adrenergic receptors are mostly located in vascular smooth muscle (VSM) (1), while beta-1 adrenergic receptors mainly located in the heart, beta-2 in the smooth muscles of blood vessels & airways and beta-3 receptors in the subcutaneous adipose tissue (2). Therefore, the modulation of sympathetic discharge from these receptors is responsible for vascular smooth muscle tone.

Phenylephrine is a well-known vasoconstrictor which is classified as an alpha adrenergic agonist (1). The mode of action of phenylephrine is by binding with alpha 1 adrenergic receptor causing the activation of phospholipase C (PLC) which then mediates conversion of phosphatidylinositol diphosphate (PIP2) to two molecules – Inositol triphosphate (IP3) and Diacyglycerol (DAG). The inositol triphosphate which is formed in turn acts on the IP3 receptor located on sarcoplasmic reticulum. IP3 receptors are ligand-gated calcium channels which release calcium when they are activated by IP3. The binding of calcium to calmodulin to form a calcium-calmodulin complex will cause activation of MLCK (myosin light chain kinase) which then phosphorylates myosin proteins and causes vascular smooth muscle contraction.

Diacylglycerol serves to activate protein kinase C (PKC) which produces an inhibition of MLCP (myosin light chain phosphatase), thereby preventing vascular smooth muscle relaxation (3). Beta adrenoceptors are Gs coupled receptors present in the smooth muscle of most blood vessels in the body. Beta adrenoceptor stimulation leads to an increase in cAMP (cyclic Adenosine Mono Phosphate) due to the activation of an enzyme Adenylyl cyclase. This cAMP then causes the phosphorylation of Protein kinase A (PKA) leading to a decrease in intracellular calcium levels and hence promoting vasodilation (4). Nitric oxide, which is an important second messenger in VSMs, can also produce vasodilation through another separate pathway. L-Arginine is the substrate for synthesis of Nitric oxide in the vascular endothelium and it is mediated out by an enzyme named eNOS, i.e. endothelial nitric oxide synthase (4). The nitric oxide thus formed can enter the VSMs and activate soluble Guanylyl cyclase (sGC), thus mediating the conversion of GTP (Guanosine triphosphate) to cyclic GMP (Guanosine monophosphate) (5). The cyclic GMP thus formed activates protein kinase G which in turn causes the activation of MLC phosphatase leading to myosin dephosphorylation and thus produces relaxation of VSM (6). Cyclic GMP is then inactivated by conversion to 5' GMP, which is mediated by the action of phosphodiesterase (5). An important condition where nitric oxide is produced in excess is septicemia which can progress onto serious complications like refractory vasoplegic shock that is resistant to treatment with vasopressors like norepinephrine and phenylephrine (7,8).

Till now, the popularly known adrenergic action on blood vessels is vasoconstriction by alpha receptors and vasodilation by beta adrenergic action. However, a study on

spiral strips of small arteries from goat legs published by Raj et al shows that under certain conditions, micromolar concentrations of phenylephrine consistently produced relaxation of VSM from the baseline tone (9). Literature search revealed another study by Filippi et al who was also able to observe such vasorelaxation, but with nanomolar concentration of phenylephrine on a rat mesenteric vessel that was pre-constricted with certain drugs (10). As per the above study by Raj et al, there are three circumstances where micromolar concentrations of phenylephrine can produce vasorelaxation in spiral strips of goat arteries from goat legs. One condition involves an increase in the levels of nitric oxide which may be simulated using nitric oxide (NO) donors like L-Arginine or sodium nitroprusside (where the presence or action of these NO donors *per se* did not induce vasorelaxation). The other circumstances involved either a decrease in cGMP as evidenced by blockers of soluble Guanylyl cyclase like methylene blue or an increase in the levels of cyclic GMP which can be produced by drugs like sildenafil which block the degradation of cyclic GMP (by the inhibition of phosphodiesterase). Even in these conditions, excess cyclic GMP levels *per se* did not cause relaxation of VSMs. The levels of NO produced in the second and third circumstances are usually normal and it seems that nitric oxide is redirected to an unidentified vasorelaxant pathway. Vasorelaxation produced in the above three circumstances is blocked by a blocker of eNOS like L-NNA (Nω-Nitro-L-arginine), and is hence said to be nitric oxide-dependent and this relaxant effect is found to be independent of cyclic GMP. Another finding of this study is that a non-selective alpha receptor blocker like phentolamine was able to abolish the phenylephrine-induced relaxation in the goat arterial strips under the high nitric oxide environment created by

NO donors like L-Arginine or SNP. This suggests that the phenylephrine-induced relaxation of VSMs is mediated via alpha adrenoceptors and the same study further continues to show that such a relaxant effect is not mediated via beta adrenergic receptors, since the relaxant effect is not abolished by propranolol, a widely-used beta-blocker (9).

The aim of the current study is to identify if the alpha adrenoceptor pathway causing phenylephrine-induced vasorelaxation in the goat small artery preparations is also present in goat aorta. Since smooth muscle cells are arranged in circular and longitudinal arrangement in large arteries like the aorta, goat aorta isolated from the goat heart is made into two different kinds of preparations – longitudinal strips and transverse cylinders (rings), which are then mounted on an organ bath and immersed in mammalian extracellular fluid solution containing bicarbonate as buffer, maintained at 37˚C by a circulating water bath and aerated with carbogen (95% O_2 and 5% CO_2). In order to record vascular tension, a force transducer is connected to a PowerLab data acquisition system. Initially, a preload tension of about 0.5 grams is applied to the goat aortic preparation and the vascular tone is allowed to stabilize for 5 to 10 minutes. This is followed by addition of 100 μmol/L phenylephrine to the organ bath.

(a) If phenylephrine produces vasoconstriction in either the longitudinal strip or the transverse cylinder of aorta, then:

In the intervention group, L-Arginine or SNP is administered before the addition of PE to test the effect of PE in the presence of NO donors, in different sets of experiments.

(b) If vasorelaxation occurs on addition of phenylephrine, then:

Prazosin will be administered before the addition of PE in another set of experiments, to test if the vasorelaxation produced by PE alone or PE/NO combination is preventable by specific α1-blockade.

Any change in vascular tension detected by the force transducer is visualised using Igor pro software after offline computation and analysis was done using SPSS v23.0. The presence or absence of a phenylephrine-induced vasorelaxant pathway in the aorta is concluded based on changes in vascular tension before and after addition phenylephrine added subsequent to specific NO donors in each preparation.

AIM AND OBJECTIVES

Aim:

To determine if the alpha-receptor mediated vasorelaxant pathway described in small artery preparations is present in goat aorta too.

Objectives:

1. To test the effect of Phenylephrine (PE) on two different preparations of aorta – the longitudinal strip and transverse cylinder.

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2. If vasoconstriction occurs in either of the above cases, then to test the response of PE in the presence of NO donors, SNP and L-Arginine.

3. If vasorelaxation occurs in objectives 1a and 1b, or 2, to test if the vasorelaxation produced by PE alone or PE/NO combination is preventable by prazosin (specific α1-blocker).

REVIEW OF LITERATURE

LITERATURE REVIEW

The aorta as a blood vessel

Arteries are the major blood vessels carrying blood from the heart to different parts of the body. Based on their site and histology, arteries are classified into three main types: (a) Large sized elastic arteries, (b) Medium sized muscular arteries and (c) Small sized arterioles. Examples of large arteries include aorta, while radial artery is an example of medium sized artery and the small arterioles mainly for the end perfusion system in direct contact with the capillaries in various organs, eg. retinal arterioles.

As per histological studies, blood vessels have three main layers: an outer tunica adventitia, a middle tunica media and an inner tunica intima. The tunica media is usually the thickest layer among these and is composed of smooth muscle cells. The tunica adventitia or the tunica externa as it may be called is mainly composed of elastic and collagenous fibres. It is more developed in the large elastic arteries like aorta. The inner tunica intima contains the endothelial layer of cells which mediates many important physiological processes in the body.

The aorta, being a large artery, has a thick layer of elastic and collagen fibres in the tunica externa which is responsible for the accommodation of large amounts of pressure reflected directly from the stroke volume of the heart. This is possible by the elastic expansion of the vessel wall and recoil back to initial state when blood passes through it. The internal diameter of the aorta does not change with the volume of blood passing through it, but rather, the elastic components of the aortic wall help to maintain its stiffness and viscoelasticity. Due to such a phenomenon in large arteries,

they are referred to as Windkessel vessels, since they demonstrate the Windkessel effect.

The sheer thickness of the walls of the aorta mandates blood supply by various smaller vessels called the vasa vasorum. The aorta is also the site of various chemoreceptors and baroreceptors which send signals regarding blood pressure and pH of the blood passing through it to the central nervous system for the maintenance of homeostasis. The middle smooth muscle layer of the aorta is interspersed by various musculoelastic components that help maintain its tone through various physiological and pathological causes of stress, including shock states. Even in situations like septic shock, where the total peripheral resistance falls due to vasorelaxation of peripheral small arteries due to various factors, the hemodynamic changes do not produce significant alterations in the tone or luminal diameter of the aorta. This may also be contributed in part to the heterogeneity of receptors present in the aortic tissue.

As age advances, the architecture of aortic cellular components may change and the stiffness might increase due to atherosclerotic processes taking place. Hence, numerous studies have shown that the response of the aortic tissue and its receptors to external factors will decline with age.

Vascular smooth muscle

The middle layer in the wall of blood vessels is made up of smooth muscles. The contraction and relaxation of this vascular smooth muscle (VSM) regulates blood flow to various organs by altering the diameter of the lumen of the blood vessel. Vascular smooth muscle requires calcium in order to contract and the source of calcium for

such a process is the sarcoplasmic reticulum. Calcium concentration inside the sarcoplasmic reticulum of VSMs is regulated at subcellular sites in the cell membrane. The tone of VSM is predominantly regulated by the sympathetic nervous system. In order to modulate the contractile status of vascular smooth muscle, different pharmacologic agents act on specific receptors to either increase or decrease the concentration of calcium released from the sarcoplasmic reticulum (11,12).

Adrenergic receptors on vascular smooth muscle

Both alpha and beta adrenoceptors are present on vascular smooth muscle. In 1933, during his research on the sympathetic nervous system, W.B.Cannon described the presence of two chemical transmitters called sympathins in the body – sympathin E which was excitatory and sympathin I which was inhibitory. Subsequently in 1948, Raymond Ahlquist proposed that the action of adrenaline took place on two distinct receptors, alpha and beta, in order to explain the dual effects of excitation and inhibition of the same sympathetic mediator in the internal bodily environment. Ever since, the concept of receptor theory of mediation of molecules in the body was widely accepted and established in the scientific community (13).

In the sympathetic division, alpha adrenergic receptors were considered as a homogenous group of receptors until 1974. It was later proposed that alpha adrenoceptors could be classified into alpha 1 and alpha 2 based on differences in potency to bind with an alpha adrenoceptor antagonist named phenoxybenzamine. In the mid-1980s, it was found that alpha 1 adrenoreceptors also show varying affinities for an adrenergic agonist, oxymetazoline and antagonists of the receptor, WB4101 and phentolamine which then led to the concept that there are three subtypes in the alpha 1

receptor. Thus, the alpha 1 adrenoceptor was further divided into three: alpha-1A, alpha-1B and alpha-1D. All these three receptors showed high affinity to the alpha 1 receptor antagonist, prazosin. Alpha-1L is another subtype of alpha 1 adrenoceptor which was discovered to have a low affinity for the drug, prazosin. Alpha-1L may be the receptor subtype that is predominantly responsible for the contraction of prostatic smooth muscle in response to noradrenaline. Based on the above action, alpha 1 receptor antagonists are widely used in the treatment of conditions like benign prostatic hyperplasia.

Beta adrenoceptors are further classified into 3 main types – beta-1, beta-2 and beta-3. The beta 1 adrenoeceptor is predominantly found in the heart, beta 2 receptor is mainly found in vascular & bronchial smooth muscle, while beta 3 is present in adipose tissue. Normally, about 80% of beta receptors expressed in the human heart are beta 1 and the remaining 20% are beta 2 adrenoceptors. Endogenous catecholamines like norepinephrine in the circulation selectively have more action on beta 1 adrenoceptors than on beta 2 receptors. In heart failure, the ratio of beta 1 and beta 2 receptors become almost equal since there is specifically a down-regulation of the beta 1 receptors. Beta 3 receptor present on adipose tissue is mainly concerned with metabolic regulation.

Signaling pathways of vascular adrenoreceptors

Alpha adrenoceptor activation is known to cause vasoconstriction and the activation of beta adrenoceptors results in vasodilation (14). Adrenergic receptors have a seven-helix transmembrane protein structure and are G protein coupled receptors (GPCR) which has. The intracellular partners of GPCRs are G proteins, which have α, β and γ

subunits arranged in a heterotrimeric structure. Activation occurs after an agonist drug binding with GTP and this action is terminated by an intrinsic GTPase activity. This cycle of activation-inactivation is also regulated by the regulators of G-protein signalling. Once GTP is bound, Gα subunit separates from the β and γ subunits to produce downstream effects. Gα subunit is further divided into $G\alpha_q$, $G\alpha_s$ and $G\alpha_i$ based on the action on various effectors. $G\alpha_q$ produces downstream effects by activation of phospholipase C, $G\alpha_s$ by the stimulation of adenylyl cyclase activity and the $G\alpha_i$ does so by the inhibition of adenylyl cyclase activity. The Gβγ subunit is involved in regulation of kinases like small G proteins mitogen activated kinases. The Gβγ subunit recombines with the Gα subunit to form a heterotrimeric structure, which takes place once the G protein gets inactivated by its intrinsic GTPase activity, where there is replacement of GDP (guanosine diphosphate) for GTP (15).

The alpha 1 adrenoceptor is a G_q coupled receptor (16). The drug phenylephrine is an example of a specific alpha 1 adrenergic agonist and it is also a known constrictor of VSM (1). Agonists like phenylephrine, on binding with G_q coupled receptor, activates phospholipase C which then converts PIP_2 (phosphatidylinositol diphosphate) to IP_3 and DAG. IP_3 binds to the IP_3 receptors located on the sarcoplasmic reticulum of vascular smooth muscle. Being a ligand-gated calcium channel, its binding with inositol triphosphate releases calcium into the cytosol. Cytosolic calcium increases by 2 mechanisms – one is, by the release of intracellular stores of calcium from the sarcoplasmic reticulum and the second mechanism is by the entry of extracellular calcium via receptor-operated calcium channels. This calcium then binds to calmodulin and forms a complex that activates

myosin light chain (MLC) kinase which further phosphorylates the 20-kDa light chain of myosin, thereby allowing myosin to interact with actin. Such an actin-myosin interaction enables the contraction of VSMs. This kind of elevation in the concentration of calcium intracellularly is a transient event and contractility is regulated by small G proteins like Rhokinase. RhoGEF (Guanine exchange factor) converts the inactive RhoA-GDP to the active form, RhoA-GTP, which inhibits MLC phosphatase. The dephosphorylation of myosin light chain by MLC phosphatase under normal conditions, leads to vascular smooth muscle relaxation. The inhibition of MLC phosphatase by Rhokinase will result in the contraction of vascular smooth muscle. This calcium-sensitizing mechanism due to the activity of Rhokinase is activated about the same time of activation of phospholipase C. The DAG that is formed earlier serves to activate protein kinase C which binds to calcium causing inhibition of MLC phosphatase activity and thereby promoting smooth muscle contraction. A decreased intracellular calcium concentration and the stimulation of MLC phosphatase will promote relaxation in VSMs (3,17).

Beta adrenoceptors on the other hand, are coupled to G_s protein. Beta adrenoceptor agonists like noradrenaline bind to the G_s protein and stimulates the activity of adenylyl cyclase which converts ATP to cyclic AMP. Cyclic AMP then phosphorylates PKA (protein kinase A) thereby producing various downstream effects by the phosphorylation of different proteins. Among the downstream effects, one is to cause a decrease in the intracellular concentration of calcium and hence promote vasodilation in blood vessels. Some of the other effects include the modulation of myocardial contractility in the heart, alteration in mitogenic and proapoptotic

functions of the beta adrenoceptor pathway. Beta 2 receptor was discovered to be coupled to G_i protein in addition to G_s/Adenylyl cyclase/PK-A, in contrast to beta 1, which is not coupled to G_i protein. Here, the consequence of this difference in coupling is that, the stimulation of beta 1 receptor was found to be proapoptotic in cardiomyocytes, whereas the stimulation of beta-2 receptor was not. It was also found that in transgenic mouse models with overexpression of beta-2 receptors, the myocardial performance in the mice was significantly improved (13,14).

Alpha-2 adrenoceptors are coupled to Gi proteins on VSMs. The preceding effect is the inhibition of adenylyl cyclase activity and therefore a decreased formation of cyclic AMP (18). This decrease in cyclic AMP levels produces constriction of VSMs (19). Thus, the activation of either alpha 1 or alpha 2 adrenoceptors on vascular smooth muscle will produce vasoconstriction (20). The alpha-2 receptors are further classified into three main subtypes – alpha 2A, alpha 2B and alpha 2C. All the three subtypes produce their effects via inhibition of cyclic AMP. The alpha 2B receptor is predominantly present in the smooth muscle of peripheral vessels and thus mediates vasopressor effects. The alpha 2A and 2C subtypes are present in the central nervous system and the stimulation of these receptors may produce analgesia, sedation and sympatholytic effects. The sympatholytic effects of alpha-2 adrenoceptors is due to the fact that pre-synaptic alpha-2 receptors in the central nervous system inhibit the continued release of neurotransmitters like noradrenaline by negative feedback mechanisms. Here, the reduction in cyclic AMP prevents calcium ions from entering into the nerve terminal, thereby producing a feedback inhibition of noradrenaline release. The presence of this type of sympatholytic action in the CNS allows alpha-2

agonists like clonidine to be used in the clinical management of hypertension and effect a lowering of arterial blood pressure. Also, alpha-2 receptors inhibit nociceptive acticity in the neurons of the spinal cord. Hence, alpha-2 agonists may also be used in the management of chronic pain disorders (21).

Role of nitric oxide in smooth muscle relaxation

Nitric oxide is an important signalling molecule that mediates various physiologic functions. It was named as the 'Molecule of the Year' in the year 1992. Three scientists, Ferid Murad, Louis Ignarro and Robert Furchgott were awarded the Nobel Prize in Physiology or Medicine in 1998 for their discovery that nitric oxide was a natural signalling molecule that mediates various cardiovascular functions. Nitric oxide is produced by the endothelium and initially, Robert Furchgott referred to it as the 'endothelium-derived relaxation factor' (EDRF) and it was studied extensively in order to be characterized. The synthesis of nitric oxide is carried out by an enzyme family called the nitric oxide synthases (NOS) which converts the aminoacid L-Arginine to L-citrulline and nitric oxide in the blood vessels. NOS was found to be of three different types – the endothelial nitric oxide synthase (eNOS), the neuronal nitric oxide synthase (nNOS) and the inducible type of nitric oxide synthase (iNOS). Although the synthases, nNOS and eNOS were named based on their discovery in neuronal and endothelial tissues respectively, they are also widely expressed in various other tissues. Thee form of nitric oxide produced by eNOS is mainly responsible for relaxation of VSMs while nitric oxide produced by nNOS in non-adrenergic non-cholinergic neurons acts as a neurotransmitter. The iNOS, which is

mainly expressed by inflammatory stimuli, produces nitric oxide to help the body's immune system in fighting against microbial pathogens but also shows cytotoxic effects. The isoforms of eNOS and nNOS are both expressed constitutively and possess low basal activity in the body. The nitric oxide synthases are activated by the influx of calcium into cells and by the formation of calcium/calmodulin complex. NOS are also regulated by several other mechanisms including changes in nitrosylation, transcription, phosphorylation, etc. (5,22).

The nitric oxide that is produced by the endothelium of blood vessels enters the adjacent VSMs and activates sGC 100-200 fold, by tightly binding onto the heme moiety present in the beta subunit of soluble guanylyl cyclase. This activation of the sGC enzyme leads to the conversion of GTP to cyclic GMP, which in turn activates PKG I (protein kinase G-I). The PKG enzyme family includes the likes of two main types – PKG I and PKG II. Among these, PKG I is associated with the sGC/cyclic-GMP signaling pathway. This PKG also causes further phosphorylation of various proteins and brings about a host of distinct physiologic effects. Out of all of them, one such action of PKG is the activation of MLC phosphatases which causes the dephosphorylation of myosin, thus preventing the interaction of myosin and actin, leading to relaxation in VSMs. Cyclic GMP is inactivated by conversion to the redundant 5'-GMP form by the action of phoshodiesterase-5 enzyme (PDE5). Therapeutic drugs like sildenafil, which is used in the medical management of conditions like pulmonary hypertension and erectile dysfunction, acts by inhibiting this PDE5 enzyme, thereby producing vasodilation due to an increase in the levels of cyclic GMP and also by the enhancement blood flow or a decrease in vascular

resistance through the vessels. Another drug named glyceryl trinitrate, which is widely used in the clinical therapy of angina pectoris also produces vasodilation and improves coronary blood flow by the release of NO in the coronary arteries, which acts via the soluble GC/cyclic GMP pathway. Thus, the relaxation of VSMs produced by nitric oxide is stated as a cyclic GMP-dependent mechanism (5,6).

NO-dependent vasorelaxation requires alpha adrenoceptor activation

Although it is a well-known fact that the activation of alpha adrenoceptors will produce constriction of VSMs, the Plos One paper by Renu et al proposed that the relaxation produced by the alpha adrenergic agonist phenylephrine in goat artery strip VSMs, in the presence of high or normal NO levels was cGMP-independent and that it also requires the activation of alpha adrenoceptors. Such a relaxation from the base-line tension was observed with micromolar concentration of phenylephrine (9). Another study by Filippi et al also reported vasorelaxation produced by phenylephrine, but using nanomolar concentration of phenylephrine and it must be noted that it was observed in a rat mesenteric vessel which was pre-constricted with adrenaline. This relaxation of VSMs was proposed to be due to the activation of NOS due to the intracellular mobilization of calcium, and was produced by activation of alpha adrenoceptors (10). In the publication by Renu et al, three different circumstances were noted, under which phenylephrine produced relaxation in VSMs. One circumstance was when phenylephrine was added in the presence of excess NO. This high nitric oxide environment for experimentation was created in the organ bath by adding NO donors like L-Arginine or sodium nitroprusside (and it must be noted

that the NO donors, L-Arginine and SNP *per se* produced no change in vascular tone). In the other two circumstances, one involved a decrease in cyclic GMP by adding blockers of soluble GC like Oxi-diazolo-Quinoxalinone (ODQ) or Methylene blue and the other one involved an increase in cGMP by addition of PDE5 inhibitors like sildenafil – Nitric oxide levels are expected to be normal under these latter two conditions. Out of Methylene blue, ODQ or sildenafil, neither of the drugs by itself produced vasorelaxation. The phenomenon of vasorelaxation occurred only on the addition of phenylephrine. These results suggest that the phenylephrine-induced relaxation of the VSMs is independent of cyclic GMP (since the vasorelaxant effect was observed even when there was either an increase or a decrease in cyclic GMP levels) and this also requires activation of alpha adrenoceptors. Such a relaxant effect produced under various circumstances was preventable by L-NNA, a blocker of endothelial nitric oxide synthase. The summary of these results is the fact that, the phenylephrine-induced vasorelaxation is an effect that is dependent on NO but cyclic GMP-independent and also requires alpha adrenergic activation. Due to such a finding, the mechanism that was proposed in that study is that NO may have been diverted to a unique putative pathway (like in the case of sildenafil, where the excess cyclic GMP inhibited the action of soluble-GC by negative feedback and so nitric oxide may be relieved from acting on soluble-GC) and the nexus of interaction between nitric oxide and phenylephrine may be the step of inhibition of PKC. This is because it was shown that, initial activation of this protein kinase C (i.e, PKC) by phorbol-myristate-acetate (PMA) or phenylephrine at the beginning of the experiment prevented any further relaxation produced by the PE/NO combination (9). An

endothelium dependent mechanism of vasorelaxation by such alpha adrenoceptor activation has also been reported in other studies on rat pulmonary artery and rabbit bronchial artery, where it is suggested that alpha receptor activation induced nitric oxide release and this was prevented by NOS inhibitors. Constriction and relaxation mechanisms constantly negatively modulate each other at the level of the vascular smooth muscle and thus contribute to the overall vascular tone of vessels (23,24).

Adrenergic receptors producing vasorelaxation

As mentioned earlier, alpha adrenergic receptors are divided into two main types – alpha-1 and alpha-2 adrenoceptors. Alpha-1 adrenoceptors are further subdivided into three subtypes – alpha 1A, alpha 1B and alpha 1D. It is known that VSM contractility is mediated by alpha 1 adrenoceptor activation. Studies on rabbit abdominal aorta have already shown that alpha 1A is the most potent among these, while alpha-1B and 1D adrenoceptors are less effective at producing contraction in VSMs (25). The subtype of alpha 1 receptor responsible for alpha induced vasorelaxation under high nitric oxide environment had to be delineated.

A search of the literature available reveals that the publication by Renu *et al* showed that the vasorelaxation induced by phenylephrine under high nitric oxide environment through alpha adrenoceptors, was inhibited by the drug phentolamine, which is a non-specific blocker of alpha receptors. The study also reiterated that such relaxant effect was not mediated through beta adrenoceptors since the vasorelaxation was not inhibited by a beta receptor blocker like propranolol (9). Another study published by Filippi *et al* provides evidence that the alpha-1D receptor is the subtype of alpha adrenoceptors which is involved in vasorelaxant mechanisms caused by the

alpha adrenergic agonist, phenylephrine at nanomolar concentrations in the mesenteric vessels of rats. As per the study, alpha-1D receptor activation results in stimulation of PIP_2 and thereby results in the mobilization of calcium from IP3-sensitive calcium stores of the sarcoplasmic reticulum. The calcium which is thus mobilized then stimulates NOS which leads to formation of nitric oxide, thereby producing relaxation of VSMs by soluble-GC/cyclic-GMP pathway. But however, in that study, micromolar concentration of the same drug phenylephrine produced vasoconstriction and it was found to be mediated through alpha-1A receptor subtype. Such a phenylephrine-induced vasorelaxation is noted to be dependent on the ability of the endothelium to produce NO, since the relaxation is prevented by an inhibitor of NOS, L-NAME and such a phenomenon is not seen in vascular preparations that have been denuded of endothelium. Thapsigargin, an inhibitor drug of calcium-ATPase channels in the sarcoplasmic reticulum, inhibits this endothelium-dependent relaxation, suggesting the involvement of IP3 sensitive calcium stores from the sarcoplasmic reticulum in this phenomenon (10,26).

Evidence from a study conducted by Andrade *et al* shows that vasorelaxant effects may be mediated through alpha-1 adrenoceptors and also shows that alpha-1D receptor subtype is responsible for relaxation induced by phenylephrine in carotid arteries of rats. Such a vasorelaxant effect depends on endothelial production of nitric oxide and not on the production of prostanoids. The existence of such a vasorelaxant effect induced by the presence of alpha-1D receptor in these vessels serves as a local control mechanism that may help modulate the vasoconstrictor response to circulating sympathomimetic amines. The above study also set forth the fact that there may be an

impaired vasorelaxation by alpha-1D receptor and so there might be an enhanced constrictor response of VSMs to alpha-1 adrenoceptor agonist drugs like phenylephrine in conditions like hyperhomocysteinemia, which is well-known as a risk factor for various cardiovascular diseases. The hyperhomocysteinemia model was created in rats using a homocysteine-rich diet and the animal's carotid artery was used for isolated vessel experiments. Such an increased vasoconstrictor response is due to the decreased bioavailabilty of NO and an impaired superoxide dismutase activity leading to the production of superoxide radicals in blood vessels. There were no pathological or morphological changes between the vessels of the control and the hyperhomocysteinemic rat on optical microscopy. Since endothelial dysfunction is a precursor factor in the development and progress of many vascular diseases like atherosclerosis, this paper goes further to show that alpha-1D receptor induced vasorelaxation was impaired during the early stages of hyperhomocysteinemia and this led to an enhanced vasoconstrictor response (27). A similar concept was also shown by Pernomian *et al*, where a balloon catheter injury abolished phenylephrine-induced relaxant responses, which then led to an enhanced contractile response to phenylephrine later in the rat carotid artery. The mechanism proposed for this was that the cyclooxygenase-2 (COX-2) pathway generates superoxide anions which caused inactivation of nitric oxide and hence impaired the nitric oxide-dependent relaxation induced by phenylephrine (26).

Oscillatory vasomotion in vascular beds

Vasomotion refers to the spontaneous changes in the diameter or tone of blood vessels produced due to relaxation and contraction of vascular smooth muscle. This

vasomotion is present in all vascular beds which is both in-vitro and in-vivio, and it refers to the vascular tone oscillations with frequencies in the range of 1 to 20 per minute. The concept and theories of vasomotion had been put forth and described nearly 150 years ago in the wings of bats. There are three types of mechanisms responsible for cellular oscillations. One mechanism proposed for the same is an oscillatory release of intracellular stores of calcium from sarcoplasmic reticulum (called as cytosolic oscillator), while a second mechanism refers to oscillations that are produced due to ion channels in the sarcolemma (called as membrane oscillator) and the third mechanism refers to an oscillation of glycolysis (called as metabolic oscillator). The experimental evidence for the latter two mechanisms is less and the cytosolic oscillator is considered to be the most important mechanism. Based on this knowledge, it must be kept in mind that the oscillations that are produced in individual smooth muscle cells need to be in synchronization as a whole, in order to achieve a macrovascular oscillation of vascular tone (28,29). The release of the intracellular stores of calcium from the SR (sarcoplasmic reticulum) produces calcium waves and these waves are found to be absent if the sarcoplasmic reticulum calcium ATPase pump (SERCA) is blocked. These calcium waves are normally present even if extracellular calcium is absent, but they will eventually disappear, since the calcium stores from the sarcoplasmic reticulum have to be refilled by the cell membrane calcium channels. In this scenario, the SERCA pump's role serves to actively remove calcium from the cytosol and replenish the stores of calcium for the next contraction. Sodium/calcium exchangers (NCX) and PMCA (plasma membrane calcium ATPase) are also involved in calcium removal from the cytosol. This type of oscillation in

calcium waves due to the activity of the SERCA pump is called cytosolic oscillator and it contributes to vasomotion. In vascular smooth muscle, when agonists are used to induce calcium waves, calcium gets released from IP3-sensitive channel through caffeine and ryanodine sensitive receptor. Information which is decoded by different transcription factors and various oscillations which lead to expression of different proteins are found in the amplitude and frequency of calcium waves. Yet another type of oscillation is seen in VSMs due to the membrane oscillator which is present even after SERCA and release of calcium from ryanodine sensitive channels has been blocked. Oscillations in membrane potential due to the interaction between voltage-dependent calcium channels and large conductance calcium activated potassium channels are responsible for the membrane oscillator. Calcium oscillations have also been reported in endothelial cells (28). There are three homologous genes responsible for the encoding of SERCA pump namely, SERCA 1 to 3. It is also known as the housekeeping pump since it plays an important role in refilling the sarcoplasmic reticulum stores of calcium.

Among these isoforms, SERCA1 is predominantly found in fast-twitch skeletal muscle. SERCA2a is found primarily in slow-twitch skeletal muscle and in the heart whereas SERCA2b is found ubiquitously and is most significantly seen in smooth muscle tissue. The importance of SERCA pump in regulation of smooth muscle contractility can be studied using drugs that inhibit the SERCA pump, cyclopiazonic acid and thapsigargin. A 52 aminoacid chain phosphoprotein, Phospholamban, is found to be an important modulator of the SERCA pump. The unphosphorylated monomeric form of Phospholamban, will inhibit SERCA while the pentameric

phosphorylated form by the action of CaMKII (calmodulin-dependent protein kinase II) relieves the inhibition and the affinity of SERCA pump to calcium ions increases. Various studies which have been done in aorta, bladder, portal vein and gastric antrum using phospholamban gene knockout transgenic mice and possessing mutations in the calcium clearance system have been found to lead to different smooth muscle pathologies (30).

Calmodulin dependent calcium ATPase is also called as PMCA. There are four different isoforms of PMCA namely PMCA 1, PMCA 4 and PMCA 1-4 which are present ubiquitously. All these isoforms have been reported to be found in VSMs. The isoforms PMCA 2 and 3 are expressed in a cell-specific pattern. The extrusion of calcium across the sarcolemma is carried out by PMCA. They are also found to play an important role in the smooth muscle contractility of the uterus and urinary bladder. Paul and colleagues, found that half-time for the force development to KCl (potassium chloride) is prolonged in gene-targeted bladder in *pmca4-/-*, *pmca1+/-*, *pmca4-/-* and *pmca1+/-* × mice. This shows that depolarization induced calcium influx is limited by loss of *pmca4* alleles. This phenomenon may be due to the presence of sodium-calcium exchangers (NCX) found in the plasma membrane, which causes extrusion of calcium in-exchange for sodium. 20-25% of relaxation is contributed by PMCA and SERCA pumps and the remaining percentage is by the sodium-calcium exchangers. There are three main isoforms of sodium-calcium exchangers. Among the three, the most common are NCX 1.7 and NCX 1.3, which are predominantly found in the VSM (30).

The oscillations caused by the individual smooth muscle cells need to be synchronized and this will occur due to the interaction between membrane and cytosolic oscillators. Stimulation of chloride channels which are calcium-activated is possible by the calcium released from the sarcoplasmic reticulum, which causes membrane depolarization by an inward current. Gap junctions play an important role in transfer of the current generated from each cell to the adjacent electrically coupled VSM cells. This synchronized depolarization is responsible for increased calcium release from sarcoplasmic reticulum. This results from enhanced calcium influx, either due to potentiation of IP3 production by membrane-depolarization or via the L-type calcium channels. Synchronization of all the above mentioned electro-physiological events are said to be dependent on cyclic GMP. The sequential activation of smooth muscle in the beginning is unsynchronized and entrainment of active VSM cells is referred to as synchronization. Endothelium is said to play an important role in regulation of vasomotion since it is prevented by removal or denudation of endothelium in some arteries. The endothelium of such vessels might provide some amount of cyclic GMP, for the coordination of oscillators in VSMCs and this cyclic GMP is also essential for the calcium-activated chloride channels (28,31)

Studies have shown that oscillatory vasomotion can be induced by alpha-1 adrenergic agonists like phenylephrine in the small mesenteric artery of rats. The significance of this finding is that, if vasomotion can be induced in the vasculature, then it can also help play a role in the modulating the local perfusion of tissue when it is activated using sympathomimetic drugs. Also, there is evidence that vasomotion may be modulated by EDRF or nitric oxide, since the denudation or the removal of

endothelium from blood vessels resulted in an increased contractile response in the rat small mesenteric arteries on stimulation by alpha-1 sympathomimetic drugs. Sympathomimetic drugs acting on alpha-1 receptors like phenylephrine act by effecting the release of calcium and an increase in the intracellular levels of calcium in the smooth muscle. This calcium can then reach the cells of the endothelium from the VSMCs by diffusion through myoendothelial gap junctions. The myoendothelial gap junctions also play another role. The calcium that reaches the endothelial cells stimulates the opening of certain K+ (potassium) channels which are calcium-activated, which causes a hyperpolarization of the cell membrane. Once hyperpolarization occurs, the change in membrane potential will be conducted back to the VSMCs via myoendothelial gap junctions. Overall, this phenomenon by which hyperpolarisation occurs by the change in membrane potential is referred to as the EDRF-dependent component of oscillation. Thus, alpha-1 sympathomimetic agents can be used to induce oscillatory vasomotion and the practical use of this phenomenon is to help maintain intestinal perfusion, particularly in patients who have some form of circulatory shock and are treated using alpha-1 agonist drugs. Thus, the vasoconstriction induced in the small mesenteric arteries of rats van be modulated by the endothelial system of cells and at higher concentrations, it is also found that it can produce oscillatory vasomotion. Such a phenomenon of oscillatory vasomotion is shown to be mediated atleast in part, by the EDRF (32).

Alpha adrenergic agonists in the treatment of septic shock

Sepsis is one of the leading causes of death in critically ill patients and it is due to the initiation of a large host of uncontrolled inflammatory responses by the body. Here,

the important pathophysiology in sepsis is the overproduction of one or more secondarily induced host mediators. A large number of studies conducted on both humans and animals show that TNF α and IL-1, which are anti-cytokine agents, are the principal toxins which are secondarily induced in the host as mediators of the inflammatory response. But clinically and from a therapeutic standpoint, none of these anti-cytokine agents proved to be useful or successful in the treatment of septicaemia in various trials. The recombinant form of activated protein C was found to have anti-thrombotic, pro-fibrinolytic and also some anti-inflammatory properties. This form of recombinant APC also reduced relative risk of death by relatively by 19.4% and decreased the absolute risk by 6.1%, but the main side effect is that it has an higher risk of developing bleeding manifestations (33,34). A major complication of sepsis is septic shock, which is characterized by hypo-tension and vascular collapse. It is believed to occur as a result of cytokine dependent induction of the inducible nitric oxide synthases (iNOS), which in turn leads to excessive NO production in-vivo which can then lead to pathological vasodilatation of arteries and thereby cause extensive tissue damage. Such a drastic chain of events is mostly due to the release of endotoxin that is present on the cell wall of gram negative bacteria (which can lead to endotoxic shock) but it is also possible that gram positive microbes, viruses, certain forms of fungi and parasites can also be the causative organisms. The patients who are affected are initially in a hyperdynamic circulatory state with tachycardia leading to progressive vasorelaxation at the peripheral level and this later ends up causing compromised tissue oxygenation and perfusion. Lipopolysaccharide (LPS), the component that is present in the outer cell membrane of most gram negative

pathogenic bacteria is the endotoxin which is released in septic patients. The LPS thus released is the prime mediator of such a high rate of morbidity and mortality in septic shock. The presence of LPS and other such bacterial products in the blood is detected by the immune cells of the body which causes production of cytokine agents like IL-6, TNF-α, IL-1β and IFN-γ (interferon gamma) into the circulation, leading to the condition called septic shock. The septic shock model can be induced and studied experimentally by injecting LPS into animals and they are observed for a rise in the levels of cytokines in the blood, which on detection will prove the induction of the sepsis model. The fatal connection between the overproduction of nitric oxide and the appearance of septic shock is evidenced by the fact that the fall in blood pressure in septicemic has been brought back to normal by administering NOS inhibitors in the patients with septic shock and also in the animal models of sepsis (34).

Bacterial lipopolysaccharide binds to specific proteins of the human body like LBP (LPS binding protein) and the resulting complex interacts with CD14, a cell surface molecule. There is a lot of recent evidence which shows that the transduction of signals on binding with proteins like LPS occurs across the receptors on the membrane, for example, Toll-like receptors 2. Tumor necrosis factor binds to the receptors on the cell membrane as well (P55 with type I-tumor necrosis factor, P75 with type II-tumor necrosis factor) and this binding leads to the host of inflammatory responses, proliferation of various cells and many apoptosis of other cell types. Alhough the precise mechanism of expression of inducible-type of nitric oxide synthase is not yet clearly known, it is believed that the activation of tyrosine kinase can cause the secretion of various cytokines and also cause signal transduction, as

soon as the cytokines binds with their corresponding receptor. These assumptions are based on reports of prevention of circulatory failure by the use of drugs inhibiting tyrosine kinase activity in mice. The onset of multiple organ dysfunction syndrome (MODS) indicates the inevitable progression of sepsis, since the presence of septicemia causes wide hepatocellular damage and thus causes the elevation of liver enzymes like aspartate aminotransferase which is usually found to be abnormally high. Multiple organ dysfunction syndrome occurs quite late in the progression of sepsis where there is an onset of hypotension in peripheral blood vessels and hyporeactivity of the VSMCs to vasopressor drugs at that point in time, in turn leads to the failure of the major organs like liver, lung, brain and kidney, finally causing death of the individual (34).

Renu *et al* showed that the vascular tension decreases in a goat artery strip that has been treated with L-Arginine, which is a NO donor, but it happens only when it is followed by addition of vasoconstrictor agents like phenylephrine. Considering the above observation, we conclude that administration of vasoconstrictor agents in septic shock patients have poor outcome as hypotensive situation may be worsened since it is a condition where there is high levels of nitric oxide present in the blood (9). Norepinephrine is the drug which is the recommended vasopressor in the treatment of septic shock and there are also other agents like dopamine, epinephrine, vasopressin and phenylephrine which is also used (35). A review of the literature suggests that activation of alpha-1 adrenoceptors causes increase of heart rate directly or it may cause the decrease of heart rate indirectly via parasympathetic activation. Studies also show that the addition of phenylephrine in the presence of the drug prazosin, an alpha-

1 adrenergic antagonist which inhibits the parasympathetic preganglionic alpha-1 adrenoceptors, while the former drug exerts a positive chronotropic effect on the heart via the action on alpha-1 receptors in the heart and this mechanism was also found to be partially mediated by the β receptors of the heart (36). As suggested by previous studies, drugs like prazosin, which are alpha 1 receptor antagonists, are useful additions to dopamine in the clinical management of cardiogenic shock (37). A study by conducted by Bond *et al* concluded that another drug named tiodazosin, which is also an alpha-1 adrenoceptor antagonist, was able to block the decompensatory vasorelaxation in hemorrhagic shock induced in rats by using Wiggers hemorrhagic shock protocol. It was also found that alpha 2 receptor blockade caused an accentuation in decompensation of the shock by 35%. Hence, the incorporation and use of alpha 1 receptor blockers like prazosin seems to help in the medical treatment of septic shock in critical care (38). As it is hypothesised that the activation of alpha adrenoceptor in the presence of high nitric oxide in the in-vivo can actually worsen the hypotension of septic shock, giving adrenergic agonist drugs after selectively blocking the alpha adrenoceptor in patients with septic shock may improve the hemodynamic effects in the circulation by adjusting inotropy and chronotropy of the heart and this kind of optimization in therapy can therefore put off the development of hypotension, since the contractility of the heart alone will be able to maintain blood pressure, even without the effect of peripheral resistance contributed by the vasculature.

Isolated tissue preparations to study function of VSMs

An essential tool for the study of smooth muscle function for pharmacologists and physiologists alike is the use of isolated tissue bath assays. This method is being used

for more than 100 years, and it still retains its significance today as it is still considered as the standard method to study concentration-response curve of various drug. It is also used to test and observe other smooth muscle functions owing to its simplicity, flexibility and reproducibility. This kind of tissue bath assays is also useful in the study of very small tissues ranging from a tiny murine mesenteric artery to the size of a large porcine ileum. This method is also used to study the effects of drugs based on a sequence of events like its receptor localization and interactions with other drugs, signal transduction, subsequent actions of drug-series and also to study second messenger systems. Studies using this technique have also helped scientists understand the basic modalities of therapy and have led to the discovery of drugs for various medical disorders including those for non-communicable diseases like hypertension, heart failure, diabetes, asthma and some gastro-intestinal diseases, thus forming a huge impact as an essential tool in basic medical research (39).

The most common variation of this kind of experimentation will be discussed as a modality henceforth. To initiate this kind of tissue testing, the tissue of interest to be studied has to be first isolated from the source animal or test group with minimal manipulation and has to be mounted in an organ bath. Before mounting the isolated tissue, initial preparation of the sample must be done as per the requirement of the experiment. Then, one end of the isolated tissue is fixed to the bottom of the organ bath using materials like a suture thread, onto a metal hook at the base and the other end of the same tissue will be connected to a force transducer apparatus, which is attached to a data acquisition system. The organ bath is maintained at body temperature, which is around 37°C, by means of a circulating water pump mechanism

and at the same time, it is aerated with carbogen (95% O_2 with 5% CO_2) to maintain optimal gas conditions similar to in-vivo environment. In order to further simulate a in-vivo environment, the organ bath is then filled with physiological salt solution (PSS) or with extracellular fluid (ECF) solution, as is the case in this study. It must be remembered that every tissue produces its optimum response at a certain length and this is called passive tension. Now, after the completion of setting up of the tissue within the organ bath, the data acquisition system is switched on and the tissue is stretched to its limit of passive tension. When this done, within a few minutes, the tissue will then slowly relax to a particular tension called as its resting tone. Following this period of time taken for equilibration of the tension of the tissue, the drugs required for the experiment are added to the bath at specific concentrations, such that the final concentration of the drugs in solution are adjusted based on the volume of physiological solution present in the organ bath. Following addition of the drugs, the response of the experimental tissue is recorded in the data acquisition system. The tissue's viability can then be tested by using drugs like KCl, potassium chloride, which is known to produce a contractile response in most smooth muscle tissues and this is done by adding a high molar dose of KCl at the end of the experiment. The data that is thus acquired by this method will then be processed to remove noise and the resulting values will be analysed using software such as GraphPad Prism or IBM SPSS. The advantage in this kind of technique is that the tissue is viable and it functions as a whole. So the values of the resulting physiological force-tension response (which may be either contraction or relaxation) can be extrapolated and applied to the whole body. Numerous variables that are required for the study can be

measured at any time during the experimentation process which further potentiates the use of the data incited by the experimental drug to be carried over to the body on the whole. Another advantage of this study is that, various tissues can be isolated for experimentation from the same animal and the specimens acquired from the animal can serve in the study as its own control. However, this method is not without disadvantages. One main disadvantage of this technique is that, if the isolated tissue is damaged during surgical removal, isolation, preparation or mounting for the experiment, the recording may give differing and unreliable results that will not be reproducible. A few examples of this kind of damage may be like in the scraping or sloughing away of the endothelium during the isolation of large, medium or small vessels, like arteries and veins. Therefore, keeping this in mind, the tissue that is isolated for study has to be done so without injury, or else the results may get affected. Poor water solubility of certain drugs and drugs that are hydrophobic may cause precipitation within the physiological salt solution, which in turn may also affect the outcome and results of the experiments of the study. The extent of tissue viability also varies with the amount of time period taken for the completion of the experiment and there are many other factors that may also vary with the number of precautions taken while isolating, preparing and mounting the tissue for the purpose of the experiment. The above described setup is a fundamental one and it can be changed with adjustment of various components to induce changes in various parameters of the study. An example of adjusting the setup is by the addition of electrodes to stimulate the field of the nerves innervating the experimental tissue specimen and also the addition of temperature and pH probes for the purpose of studying the effects of

changes in acidity or alkalinity and even variations of temperature on the response of the tissue to drugs used in the study (39).

Even though there are classical methods to study the effects and dose-response relation of drugs on isolated tissue using isotonic/isometric kinds of force transducers, there are quite a number of newer studies that have been described using newer methods like using image sensors to study the micro responses of the isolated smooth muscle specimen in the experiment. Such a system includes the use of a high-resolution camera with the function of a telecentric lens and the whole setup was also coupled to an illumination system for better picking up changes in signals. The responses of the tissue specimen to various factors like drugs can be calculated by the change in lumen diameter of the isolated tissue like an aortic ring. At the heart of such a system is a critical image processing algorithm that has been created over many years, which measures the lumen area and the change in diameter of the same by calculating the number of pixels that is recorded to be placed within the area of the aortic ring. Any change in the number of pixels recorded within the area of the specimen is considered to be a change in tissue response. An on-line dose-response curve will be computed based on the monitored video by specialized software simultaneously as the experiment is being carried out. The benefit of this method over classical techniques that have been used so far is only that it does not include the usage of a variety of mechanical equipment that can damage the specimens, like transducers, suture materials, organ bath, etc. Also, the amount of drugs, gases, solutions and buffers consumed for the same are far less compared to the classical techniques. Mechanical and physical damage to the tissue is also very less as

compared to the classical techniques. Dose-response curves acquired following the use of phenylephrine and recorded using the above type of image-sensor method is found to be similar to that obtained with the traditional mechanism of using force transducers in the rat large artery rings like aorta. Thus said, the cheaper, faster and more cost-effective alternative to the traditional techniques using force transducers to measure contraction-relaxation responses in experimental tissue is image-sensor method (40).

There are also other methods used to study the reactivity of VSMs to various drugs and factors. Some of these examples include the pressurized arterial myograph and wire myograph. Resistance arteries with very small internal diameters in the range as small as 60 microns can be studied using wire myograph method. The wire myograph technique is done by using stainless steel wires which are ideal for the mounting of experimental ring preparations by fixing the same to two secure supports. One stainless steel support in the wire myograph technique is attached to the micrometer which is meant for adjusting the tension of the experimental tissue specimen and an isometric transducer is attached at the other end of the support. After setting a specific internal diameter for the arterial or tissue specimen which is mounted onto the wire myograph, such that it gives maximal response at the particular diameter, drugs can be added to the setup and the tension changes over the wire supports in response to the added drugs can be recorded for the experiments. The vascular reactivity of various tissue specimens in physiological as well as pathological states can be studied with an even more precise setup having environments which closely match in-vivo condition, using a pressurized myograph technique. In this method, perfusing cannulae (glass

pipettes) are used which allows the investigator to maintain the pressure of the vessel within the lumen at level very close to physiological ones. One of the cannulae used to fix the tissue is moved so as to adjust tension within the blood vessel, while the other cannula remains fixed in the same position. First the artery is flushed to remove any obstructions to flow like clotted blood, tissue or gas bubbles using specific solutions made for perfusion, before connecting the other end of the vascular tissue to the movable cannula. If the blood vessel has any branches, they are to be sutured prior to the start of the experiment in order to prevent any leakage of the perfusing solution. In order to maintain a desired pressure, a pressure transducer is conducted to the setup and used to set the required pressure. The level of contraction possible by a blood vessel can be studied by using the apparently visible changes in the diameter of the lumen of vessels by means of a video camera that has an imaging system in its functionality.

All the above described methods may be utilized to study the reactivity of blood vessels by using different kinds of preparations, including modifications in each method as deemed necessary. Another example of modification of preparations are those where endothelium is intact and those where the endothelium has been sloughed off to remove the effects of the endothelial factors in the experimental study. In such cases, the denudation of the endothelial layer can be confirmed during experimentation by the loss of relaxant of VSMs to the addition of acetylcholine in the tissue under study (41).

Factors affecting contractility of VSMs

Numerous factors can affect the contractility of VSMs to the addition of drugs including skills in dissecting the tissue, handling of the specimen, methods of mounting the sample, the preparation of physiological salt solutions and buffers, pH and temperature of solutions used in the study, the optimal resting tension for the particular tissue, age of the animal from which the specimen has been isolated and even the time taken for isolation of the tissues. The specimen tissue for experimentation needs to be handled with so much care and in order to preventing manual damage, forceps are used for the gentle removal of the extraneous unwanted connective tissues surrounding the sample under study, without causing any undue shear stress or strain by stretch of the vessel. An important factor that can affect the results of most tissue studies is the improper constitution of the fluids used in the experimental setup. Even so, the pH of the fluid can change due to many factors during the experimentation process. In order to keep the acidity or alkalinity of the solution used for the study under check, the selection of an apt buffer is required. If a buffer is not used, this can cause changes in the pH of the solution due to which the viability of tissue can be severely affected. The pH of the solution should be maintained by allowing the bubbling of carbondioxide through it if bicarbonate buffer is used for the study. Another buffer that can be used instead of the bicarbonate buffer is the HEPES buffer, the usage of which will cause the pH of the solution to be neutralized around 7.4, by using sodium hydroxide. In order to slow down various innate metabolic processes from occurring in the dissected tissue, it is advised to lower the temperature of the fluid used for transportation and dissection. Hence, the

media used for transportation and dissection of the experimental tissue is maintained at around 4°C. Depending on the type of tissue or blood vessel specimen used and the source of the sample, the optimal resting tension needs to be adjusted to ensure maximal responses to the drugs in the study. Another crucial factor that needs to be taken care of is the amount of preload applied to the vascular specimen used in the study. Optimal adjustment of the preload applied to the tissue plays an important role in getting the best contractile response possible from the experimental tissue and hence it needs to be determined accurately. The age of the animal from which the tissue is obtained can also affect responses of the tissue to experimental drugs, since the ageing process itself can cause various structural and morphological changes in the blood vessel or tissue used in the study. Studies have shown that in the rat pulmonary artery, the responses mediated by the endothelium in the VSMs to produce a vasorelaxant response to acetylcholine shows a progressive decline with advancement of age of the experimental rats. So, even the age of animals from which samples are isolated and used for experimentation proves to be an important factor. As much as possible, the time taken for the isolation of the tissue sample and preparation for mounting and recording data needs to be kept minimal (41).

In this current study, the classical method of recording data using isometric force transducers was the method used for all the experiments and the data acquisition system used for the same was from the PowerLab brand of AD Instruments. A modified form of the protocol followed for the isolated vessel experiments in the paper published by Renu *et al* from our department, was used. In that study, small arteries from goat legs were made into spiral strips by continuous helical cuts along

the length of the isolated vessel and mounted in such a way that the long axis of smooth muscle cells in the vascular tissue is in the same plane and parallel to the spiral orientation of the prepared vessel. This kind of a preparation was used so that the contraction or relaxation of the VSMs in the experimental tissue can develop more fully. To achieve this kind of response, care must be taken that the orientation of the spiral strip should be identical between the various vessels used in the study (9,41,42).

In this study, isolated aortae from goat hearts were made into two different preparations – rings (transverse cylinders) and longitudinal strips, instead of using it as a whole segment or in spiral strip preparations as followed in the earlier study.

In the study reported by Filippi et al, in nanomolar concentration, phenylephrine was produces relaxant response in vascular smooth muscles and the subtype of receptor that causes such a vasorelaxation was alpha 1D. This phenomenon was also found to be dependent on the endothelium. The study was done using a rat mesenteric artery which was pre-contracted using U46619 which is an analogue of thromboxane, an cytokine mediator (10). But on the contrary, the Plos One from our department shows evidence that in micromolar range of concentration, phenylephrine was able to induce relaxation of VSMs from their basal tone in arterial strip from goat legs. Such a phenomenon resounds the conditions in pathological states with excess nitric oxide levels like that of septic shock and might also have therapeutic implications in therapeutic modalities of the same. Another claim by the same study was that phenylephrine in micromolar doses induces relaxation of VSMs through alpha adrenoceptors and not via beta adrenoceptor, as might be the regular norm of such vascular behaviour. The vasorelaxant response was dependent on nitric oxide but

independent of the cyclic GMP pathway (9). Therefore, alpha sympathomimetic drugs like phenylephrine causing a decrease in vascular tension under special circumstances where there is nitric oxide excess is of great concern, since the same drugs are used as a vasopressor agent to maintain blood pressure in various pathological states including conditions like septic shock. The aim of the present study is to observe if phenylephrine which could induce relaxation at micromolar doses in the goat leg arteries via some specific pathway can do the same in a large elastic vessel like aorta. The objective mentioned in this study will be achieved by adding different nitric oxide donors followed by phenylephrine into the organ bath in which different goat aortic preparations will be mounted and results will be interpreted based on the aortic tissue response of contraction or relaxation to each intervention.

MATERIALS AND METHODS

MATERIALS AND METHODS

The approval for this study was given by the Institutional Review Board (IRB) of Christian Medical College, Vellore (IRB no: 10412, dated 15.12.2016). The study and all the experiments were carried out using the CMC Fluid Research Grant from the Department of Physiology, Christian Medical College, Vellore from a period of June 2017 to June 2018.

Materials required for isolated vessel experiment

- Double-walled organ bath
- Circulating water bath set at 37°C
- Mammalian ECF solution
- White dissection board
- Iris scissor – 1 nos.
- Toothed forceps – 1 nos.
- Straight forceps – 1 nos.
- Size 11 Surgical blade – 1 nos.
- Petridishes – 2 nos. (for ECF for dissected samples)

- Silk thread
- Suture Needle – 1 nos.
- 100µl Pipette tips (for making loop)
- Micropipette with pipette tips (1000µl and 100µl) – to add drugs
- Carbogen cylinder
- Force transducer from AD Instruments
- PowerLab data acquisition system
- Laptop for recording

Fig.1. The PowerLab data acquisition system

Stock solutions for the experiment

10mM (millimolar) concentration stock solutions were prepared for all of the following drugs used in the study, except for adrenaline bitartrate which is available in ampoules as 3M (molar) concentration.

- NO donor, L-Arginine hydrochloride
- NO donor, Sodium Nitroprusside
- Alpha receptor agonist, Phenylephrine hydrochloride
- Alpha-1 blocker, Prazosin hydrochloride

The above stock solutions for phenylephrine, L-arginine, SNP, prazosin and adrenaline were prepared newly each time, on the day of experiment. The mammalian ECF solution which was prepared in bulk was stored at 4^{o} Celsius for use as and when required.

Fig.2. Force transducer from AD Instruments

Composition of normal extracellular solution

NaCl	100 mmol/L
KCl	3 mmol/L
$CaCl_2$	1.3 mmol/L
$NaH2PO_4$	0.5 mmol/L
$Na2HPO_4$	2 mmol/L
$NaHCO_3$	25 mmol/L
$MgCl_2$	2 mmol/L
HEPES buffer	10 mmol/L
Glucose	5 mmol/L

The pH of the mammalian ECF solution was titrated to 7.4 using 1M sodium hydroxide. 5 liters of this solution was prepared in bulk at a time and stored at 4°C.

Methods

Isolation of vessel

Fresh goat heart was brought from a registered slaughter house as planned earlier, on the day of experiment. The procured goat heart was then transported to the lab by immersion in a flask containing chilled mammalian ECF solution. The procedures for the planned experiment were started within 5 to 10 minutes of obtaining the heart in ECF solution. If any additional hearts or fresh aortae are available which can be used for another intervention or experiment on the same day, it was stored at 4°C and it was to be used for the same purpose within 2 to 3 hours of storage. Solutions and drugs required for the experiments are prepared and kept ready beforehand. Also, two petri dishes filled with chilled mammalian extracellular fluid (ECF) solution are kept ready before starting the dissection. Once the fresh heart was obtained, it was rinsed under running tap water and placed on a white board for dissection. Using a size-11 surgical blade, the pericardial sac was incised and the pericardial fluid was drained from the goat heart. The surrounding fat and fascia was dissected superficially, without damaging the underlying vessels. The ascending aorta was identified and traced to its point of origin. The vessel was confirmed as aorta by the patency of its lumen, as a vein would have a collapsed lumen. Additionally, a glass probe inserted through it can be felt in the apex of the heart, as it is formed by the left ventricle. Then the aorta was dissected at its base from its visible site of origin and with minimal manipulation to be as lengthy as possible, all the while trying to be as proximal to the heart as possible.

Fig.3. Fresh goat heart in mammalian ECF

After dissection, the aorta was immediately placed in a petri dish containing chilled ECF solution. The chilled ECF solution decreases the metabolic rate of VSMCs and hence prolongs the viability of tissue thus obtained and stored. After this step, the extraneous connective tissue adhering to the aorta was removed using forceps and iris scissors, while the aorta was still kept immersed in the chilled ECF solution. Viability of the isolated tissue may be affected if the specimen was not kept immersed in the chilled mammalian ECF solution. It is made sure that the aorta was completely cleared of adherent fascia and extraneous tissue as it may affect the results. After the surface of the tissue has been cleaned, the dissected segment of aorta was then gently transferred to the second petri dish which was also filled with chilled mammalian ECF

solution. The aorta specimen isolated thus far was then cut into equal sections of about 0.5 cm in length, devoid of any side branches; each 0.5 cm section was as required for one aortic ring experiment.

Fig.4. Aorta isolated in petridish with chilled ECF

Mounting the tissue

For aortic ring experiments:

A loop was made using a pipette tip of 100µl with needle and silk thread on one side of the aortic ring while a knot was made at the diametrically opposite end of the ring, leaving a longer thread, enough to be connected with force transducer. The aortic ring was then mounted in an organ bath of 25ml capacity. The loop was secured by means

of a knot to a metal hook situated at the bottom of the organ bath, while the other end of the silk thread was connected to a force transducer.

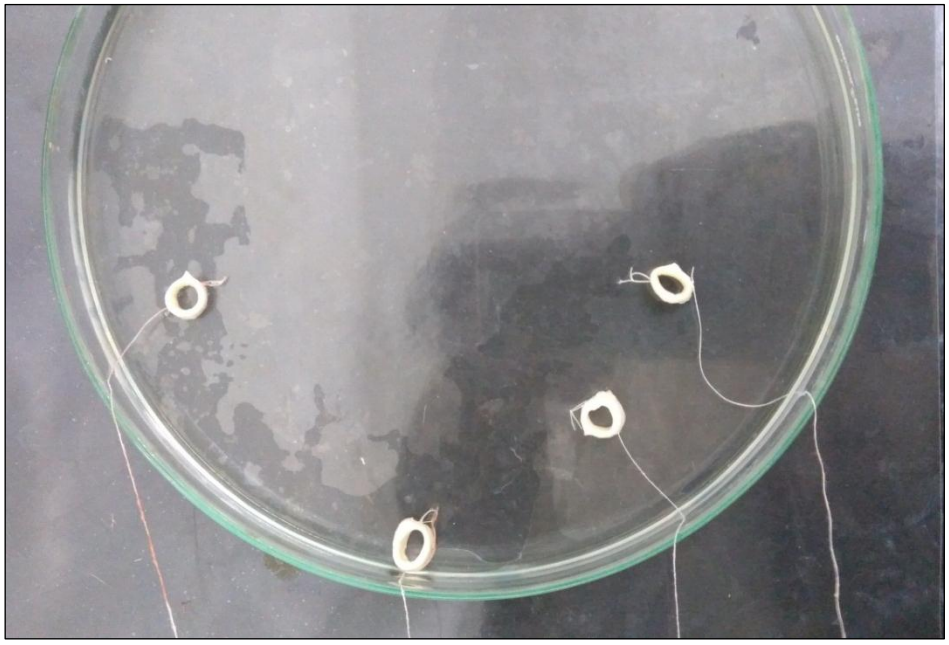


Fig.5. Multiple isolated aortic rings in petridish with ECF solution

Fig.6. Isolated aortic ring mounted in organ bath

For longitudinal strip experiments:

For longitudinal aortic strip experiments, a pair of surgical scissors was introduced into the lumen of an aortic ring preparation and a clean, straight, longitudinal cut was made. Thus, the lumen of the aortic ring was cut open into a longitudinal orientation, exposing the endothelium of vessel and thus making a longitudinal strip. A 100µl pipette tip was then used to make a loop with a silk thread and needle on one side of the longitudinal strip while a knot was made on the opposite side of the strip, leaving the silk thread long enough to be connected to the force transducer. The aortic longitudinal strip preparation was then mounted in an organ bath of 25ml capacity. The loop was secured to a hook by means of a knot at the bottom of the organ bath, while the other end of the silk thread was connected to a force transducer.

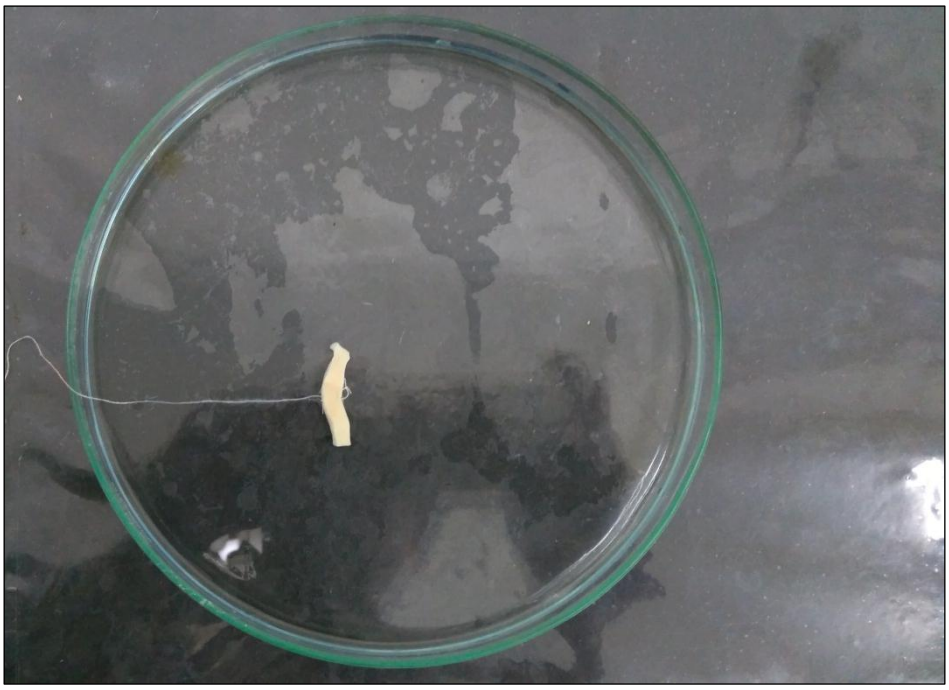


Fig.7. Isolated longitudinal strip of aorta in petridish filled with ECF solution

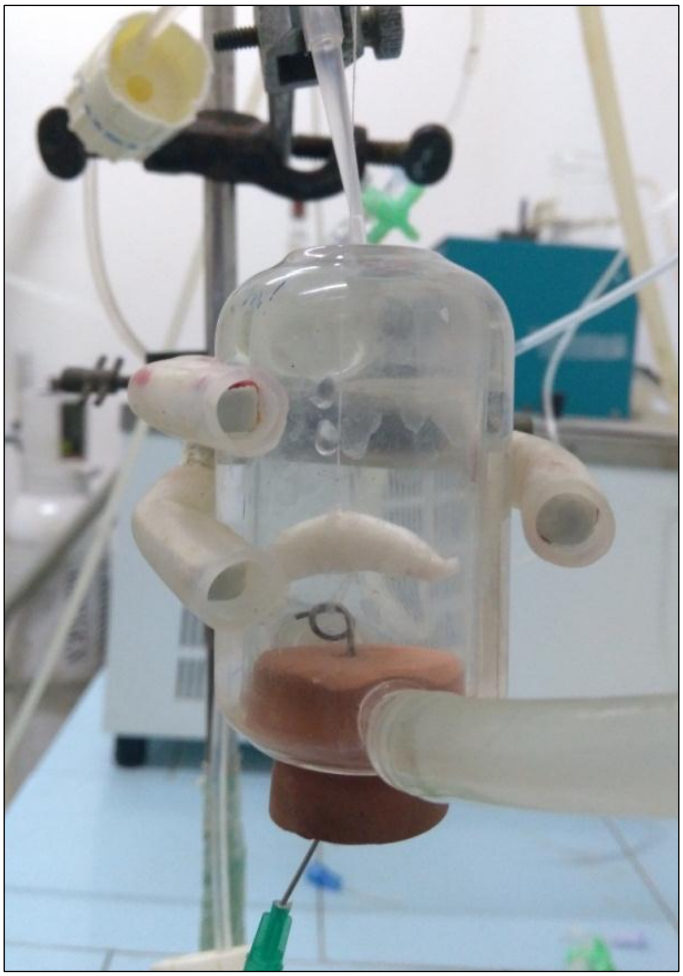


Fig.8. Isolated aortic longitudinal strip mounted in organ bath

Recording of data

The organ bath was filled with mammalian ECF solution and allowed to stay for a while so that it is maintained at a temperature of 37°C by means of a circulating water-bath and aerated with carbogen (95% O_2 and 5% CO_2). The force transducer from AD Instruments was connected to the PowerLab data acquisition system and a laptop was connected to the data acquisition system to make recordings of the experiment. If there are any remaining aortic strips or rings which can be utilized for

the subsequent experiments on the same day, they can be used within 2-3 hours of isolation and hence will be stored at 4°C in mammalian ECF solution.

The organ bath fitted on a stand, along with the height-adjustable mount for the force transducer can be seen in the following figure. A laptop pre-installed with the LabAuthor software provided by the PowerLab Company is seen with its peripherals in the picture, taken live during one of the experiments in this study while recording vascular tension using a goat aortic preparation.

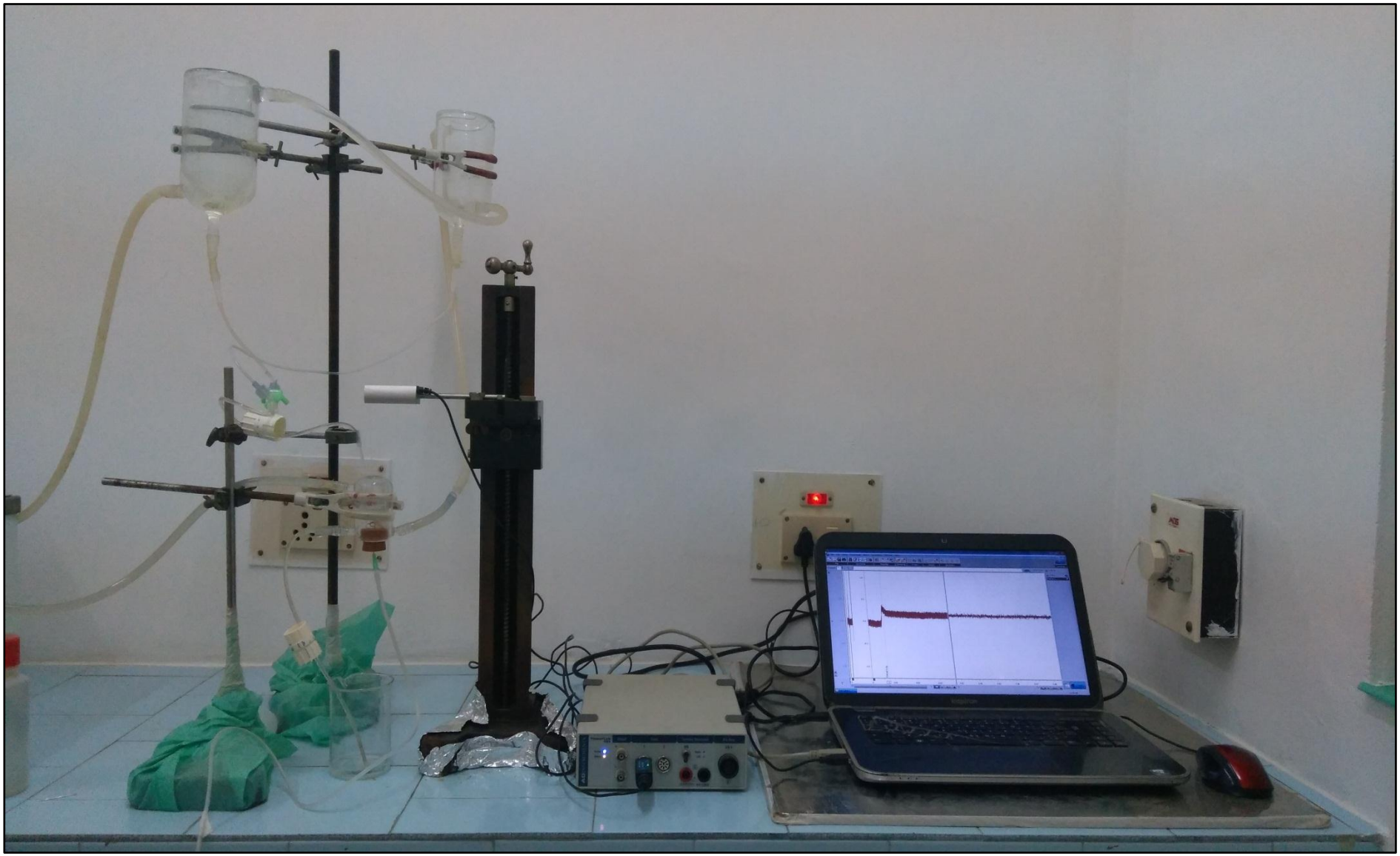


Fig.9. Complete recording setup

Solutions required for the experiments

All the salts necessary for the preparation of the mammalian ECF solution was purchased from SIGMA, which is now a part of MERCK Pharma. L-Arginine hydrochloride, Sodium nitroprusside, Prazosin hydrochloride and Phenylephrine hydrochloride were also purchased from SIGMA in HPLC grade powder or crystal form. Adrenaline bitartrate was purchased from NEON Laboratories in the form of clinical grade IV-use ampoules. 1000 times stock solution of all the above drugs purchased was prepared and an appropriate concentration of the required drug was added to the organ bath to attain the final concentration required for each experiment. L-Arginine hydrochloride, Phenylephrine hydrochloride and Sodium nitroprusside was dissolved in distilled water. Prazosin hydrochloride was dissolved in methanol.

L-Arginine hydrochloride:

The molecular weight of L-arginine hydrochloride is 210.7 g/mol. 0.02g of the drug was added to 10ml of distilled water to get a stock solution of 10mM. 1000µl of this 10mM stock solution was added to the 25ml organ bath filled with ECF to get a final required concentration of 400µmol/L for a single intervention.

Phenylephrine hydrochloride:

The molecular weight of phenylephrine hydrochloride is 203.67 g/mol. 0.02g of the powdered form of the drug was added to 10ml of distilled water to get a stock solution of 10mM. 250µl of this 10mM stock solution was added to the 25ml organ bath filled

with mammalian ECF solution to get a final required concentration of 100μmol/L for each intervention in an experiment.

Sodium nitroprusside:

The molecular weight of sodium nitroprusside is 261.92 g/mol. 30mg of the crystals form of the drug was added to 1ml of distilled water to get a stock solution of 10mM. 80μl of this 10mM stock solution was added to the 25ml organ bath filled with ECF to get a final required concentration of 400μmol/L for a single intervention.

Prazosin hydrochloride:

The molecular weight of prazosin hydrochloride is 419.86 g/mol. 10mg of the powdered form of the drug was added to 2.4ml of methanol to get a stock solution of 10mM concentration. This stock solution was stored as separate aliquots of 250μl each in separate eppendorf centifuge tubes, at -20°C. One aliquot of 250μl was added to 25ml of mammalian ECF in the organ bath, to get a final required concentration of 100μmol/L for each intervention of an experiment.

Adrenaline bitartrate:

The molecular weight of adrenaline bitartrate is 333.33 g/mol. It is available as an IV preparation in 1mg/ml ampoules, which gives a stock solution of 3M. 50μl of this 3M stock solution was added to the 25ml organ bath filled with mammalian ECF to get a final required concentration of 10μmol/L for each intervention of an experiment.

Protocol for the experiments

After the aortic preparation (ring or strip) was mounted in the 25ml organ bath filled with mammalian ECF solution, the PowerLab data acquisition system and laptop was switched on. The force transducer from AD Instruments, which senses changes in vascular tension, was connected to the PowerLab data acquisition system, also from AD Instruments. The initial calibration of the equipment was carried out using 10g weight, so as to get the recorded values in grams from millivolt. Appropriate maintenance of temperature at 37º Celsius and adequate supply of carbogen from the cylinder was ensured before starting the experiment. Initial preload was applied to the mounted tissue, in the range of 0.5gms, so that the silk thread becomes taut and then the aortic preparation (longitudinal or transverse strips) was allowed some time to stabilize to its basal resting tone. Usually, the time taken for the aortic preparation to stabilize was about 10 minutes. Once the tension of the tissue stabilized, the drug of interest was added and 5 minutes of wait time was commenced in order to notice the change in response since any change in tension could be appreciated within 5 minutes of adding the drugs. Six different set of experiments are done which includes both the control and the intervention groups. The sample size for each of the experimental groups was between four to six. In one set of experiments, after an equilibration period since the application of 0.5 grams of preload, 100μmol/L phenylephrine was added to the organ bath in each of the preparations and this was taken as the control group. Any change on adding further drugs, either constriction or relaxation detected by the force transducer was recorded as an increase or a decrease in vascular tension from the baseline respectively.

If vasoconstriction occurs on addition of PE in either of the aortic preparations (longitudinal strip or transverse cylinder), the approach for the intervention groups will be,

1. To test if the vasoconstriction induced by phenylephrine in longitudinal strips is preventable with NO donors, L-Arginine (L-Arg) or sodium nitroprusside

2. To test if the vasoconstriction induced by phenylephrine in transverse cylinder is preventable with NO donor, L-Arginine (L-Arg) or sodium nitroprusside

3. If vasorelaxation occurs in the control group (PE alone) or in any of the above interventions (PE/NO combination): To test if the vasorelaxation is preventable by prazosin (specific α1-blocker)

Immediately after the addition of each drug, a comment was added manually during the recording, which indicates the identity of the drug added and its concentration. At the end of each experiment, the ECF in the organ bath which contains the mixture of various drugs added during the experiment was drained off and refilled to capacity with new mammalian ECF solution.

In case of drugs that do not produce any kind of response, adrenaline was used to test viability of the tissue instead of potassium chloride (KCl), since studies in our lab showed that aortic preparations constricted in response to adrenaline, but KCl was ineffective in producing any tissue response to the goat aorta. The aortic tissue response to adrenaline started at 5μmol/L and maximal constriction was achieved at 10μmol/L. Hence, 10μmol/L of adrenaline was used.

If the tissue was found to be viable based on the degree of constriction produced in response to the addition of adrenaline above the baseline tone, then the changes in vascular tension of the experimental recordings was noted and the results were included in the analysis. If the addition of adrenaline does not show any increase in vascular tension above the baseline, the recordings obtained from such tissue was not included in the analysis, since the tissue was considered to be non-viable. Utmost precaution was taken not to disturb the silk thread which was connected to the force transducer while drugs were being added or when the organ bath was refilled with ECF solution for washing of the organ bath and tissue. There may be a visible increase in noise in the recorded data if the thread is disturbed during the recording. Data was recorded at a frequency of 1 kHz by using the PowerLab data acquisition system. A text file containing the values of all the readings recorded during the experiment was obtained using the data-pad option in the software. The text file was then exported to Igor pro for further processing and better graphical visualization. After the curve was smoothened to remove noise in Igor pro using the Gaussian smoothing function, a graph was obtained by plotting time (in hr:min) along the X-axis and tension (in grams) along the Y-axis. The values are noted down in an Excel spreadsheet and the significance of the results was further computed by statistical analysis using SPSS v23.0.

A sample of the goat aortae used for experimentation in longitudinal and circular preparations was taken for histological examination, to confirm maintenance of tissue architecture and intactness of endothelium.

Histology of the experimental tissues

A small piece of aortic tissue samples from each of the experimental preparations (rings and longitudinal strips) was fixed in 10% buffered formalin for a week. After fixation, tissues were dehydrated with ascending concentrations of alcohol (70%, 90%, absolute) and then cleared with xylene. The tissues were then impregnated with liquid wax for 2 hours. Paraffin blocks were made using the processed tissue.

5-micron tissue sections were made into slides and kept in oven for 1 hour to de-wax. Then, it was cleared with xylene and hydrated with descending grades of alcohol (absolute, 90%, 70%). The slides were brought to water and stained with haematoxylin for 8 minutes, washed with water and differentiated with 1% acid alcohol before immersion in Scott's tap water for blueing. The slides were then treated with 80% alcohol and stained with eosin for 30 seconds, followed by dehydration with ascending grades of alcohol (80%, 90%, absolute) and cleared with xylene. They were then mounted by a coverslip with DPX.

STATISTICAL ANALYSIS

STATISTICAL ANALYSIS

All the statistical analysis for significance of each set of experiments was done using SPSS software (version 23.0). For testing significance of an intervention within a group, the change in vascular tension before and after the addition of the drug was compared using the Wilcoxon's signed rank (WSR) test. For testing significance between two groups, the difference of the percent change in vascular tension, for example, in the presence of NO donors in intervention group was compared with that of the control group using Mann-Whitney U (MWU) test. A p-value of less than 0.05 (< 0.05) was considered as statistically significant. The results thus obtained are expressed as line diagrams and scatter plots with median. Scatter plots are done using Microsoft Excel 2010.

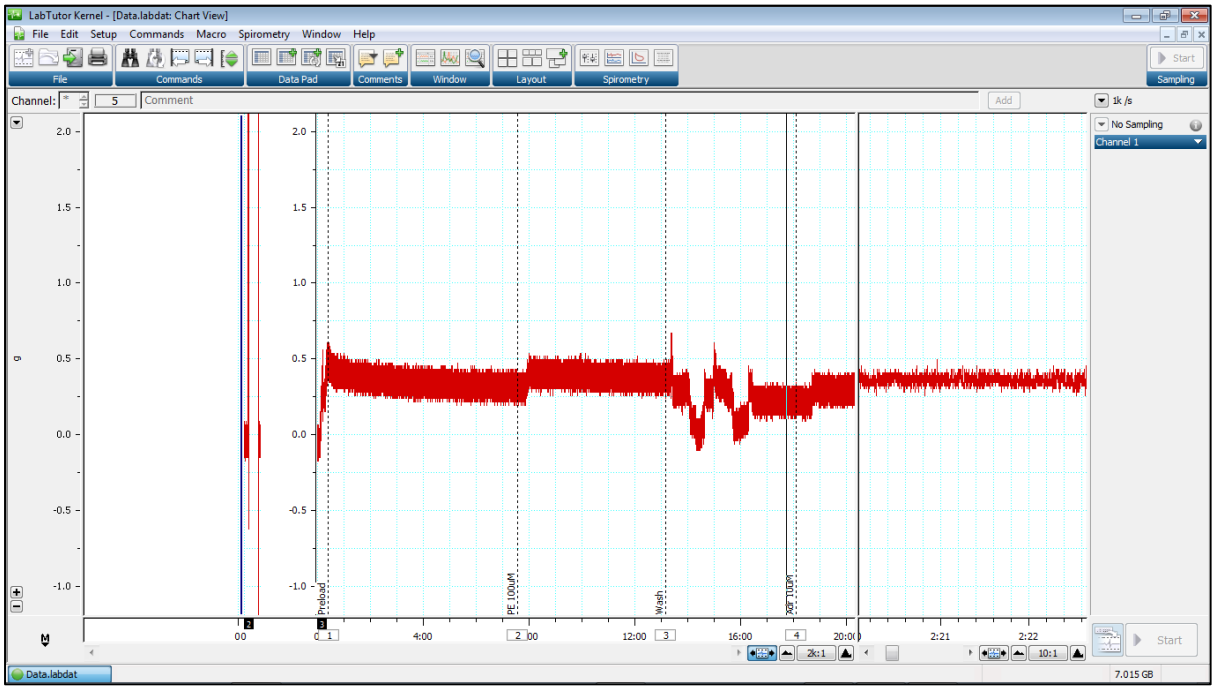
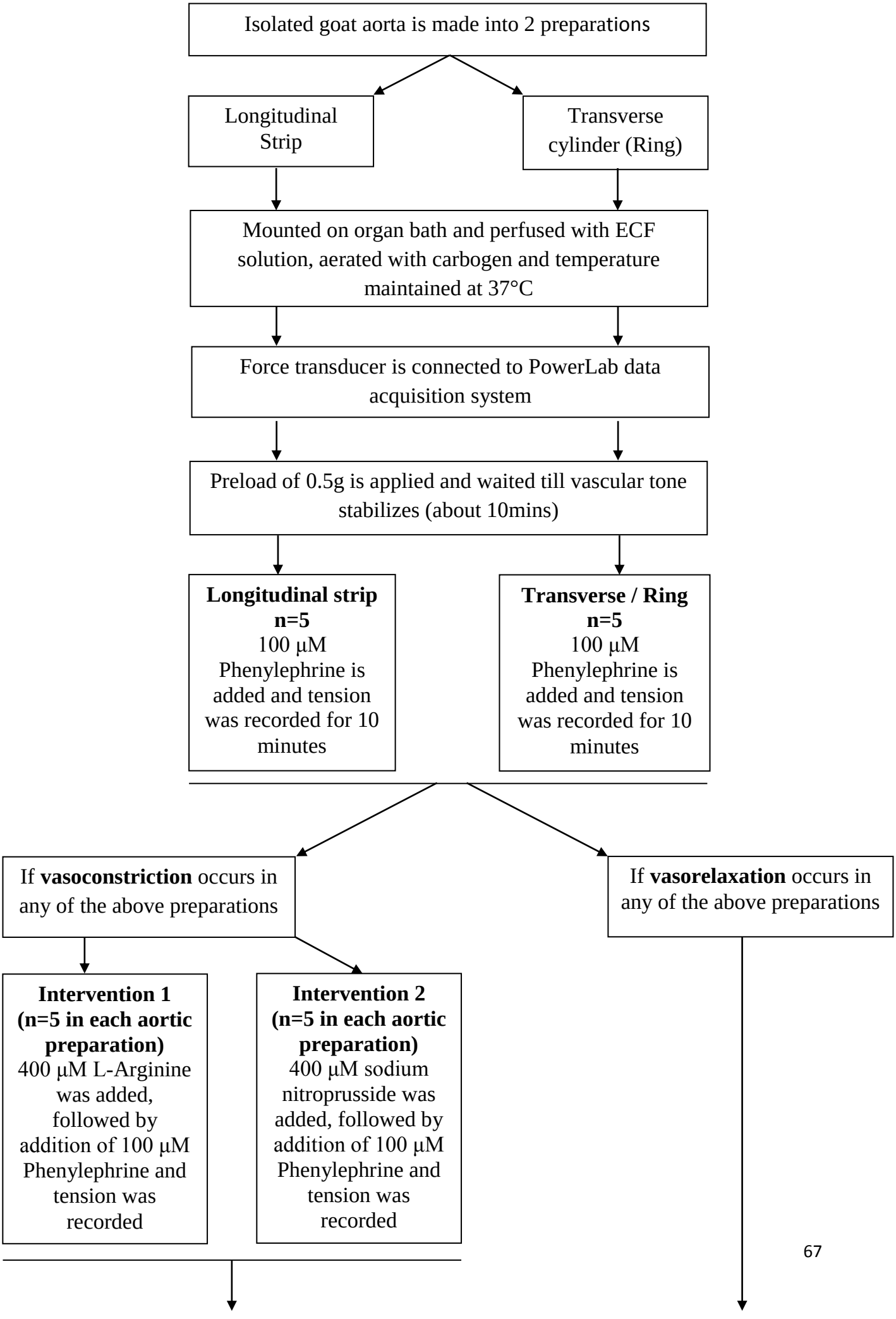


Fig.10. PowerLab data acquisition – live raw recording


Isolated goat aorta is made into 2 preparations
Longitudinal Strip
Transverse cylinder (Ring)
Mounted on organ bath and perfused with ECF solution, aerated with carbogen and temperature maintained at 37°C
Force transducer is connected to PowerLab data acquisition system
Preload of 0.5g is applied and waited till vascular tone stabilizes (about 10mins)
Longitudinal strip n=5
100 μM Phenylephrine is added and tension was recorded for 10 minutes
Transverse / Ring n=5
100 μM Phenylephrine is added and tension was recorded for 10 minutes
If vasoconstriction occurs in any of the above preparations
If vasorelaxation occurs in any of the above preparations
Intervention 1 (n=5 in each aortic preparation)
400 μM L-Arginine was added, followed by addition of 100 μM Phenylephrine and tension was recorded
Intervention 2 (n=5 in each aortic preparation)
400 μM sodium nitroprusside was added, followed by addition of 100 μM Phenylephrine and tension was recorded

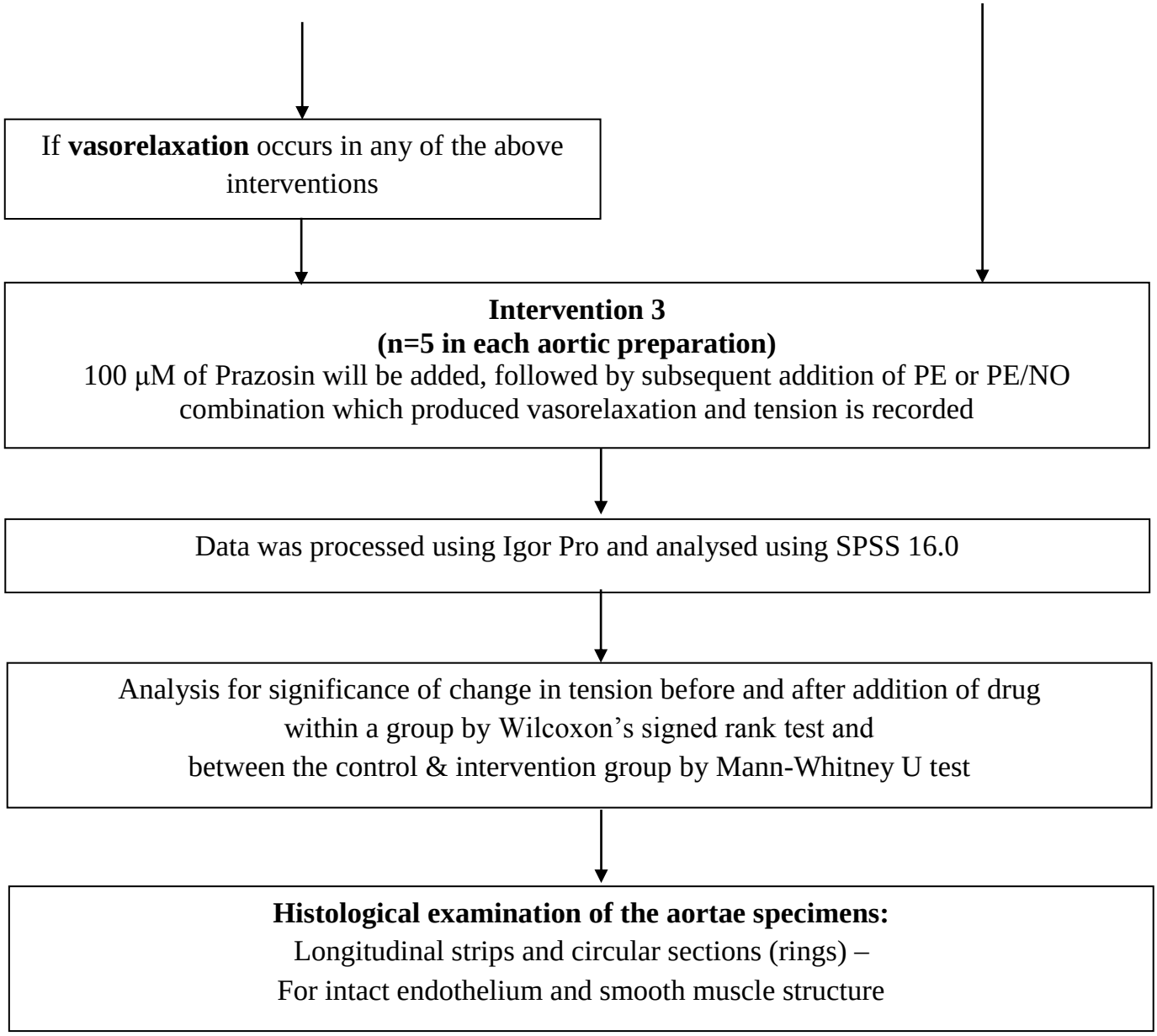


Fig.11. Detailed diagrammatic algorithm of the study

RESULTS

RESULTS

Recordings for the change in vascular tension was made using PowerLab data acquisition system at a sampling rate of 1 kHz and this data was further processed and smoothened using the Gaussian smoothening function in Igor pro for better visualization and further analysis for significance in change of tension using SPSS version 23.0.

Line diagrams and scatter plots for the graphical representation of change in tension in each group of experiments was done using Microsoft Excel 2010.

The viability of the tissue was confirmed by further by the addition of 10µmol/L adrenaline. Exposure of aortic smooth muscle to 10µmol/L of adrenaline causes PLC-mediated calcium release from the sarcoplasmic reticulum which produces maximal contraction in the tissue. Only tissues that produce an apparent vasoconstriction to adrenaline above the baseline are considered to be viable and the results of the experiment are taken into account for analysis in the study. If there is no apparent constrictor response to adrenaline, the recordings of the experiment are not used for analysis in the study.

Phenylephrine per se produced vasoconstriction in longitudinal strips under normal conditions

The addition of 100µmol/L phenylephrine produced significant vasoconstriction by an increased vascular tension from 0.31 gram to 0.43 gram (median values, n = 5) in the longitudinal strips of aorta. (Fig.12)

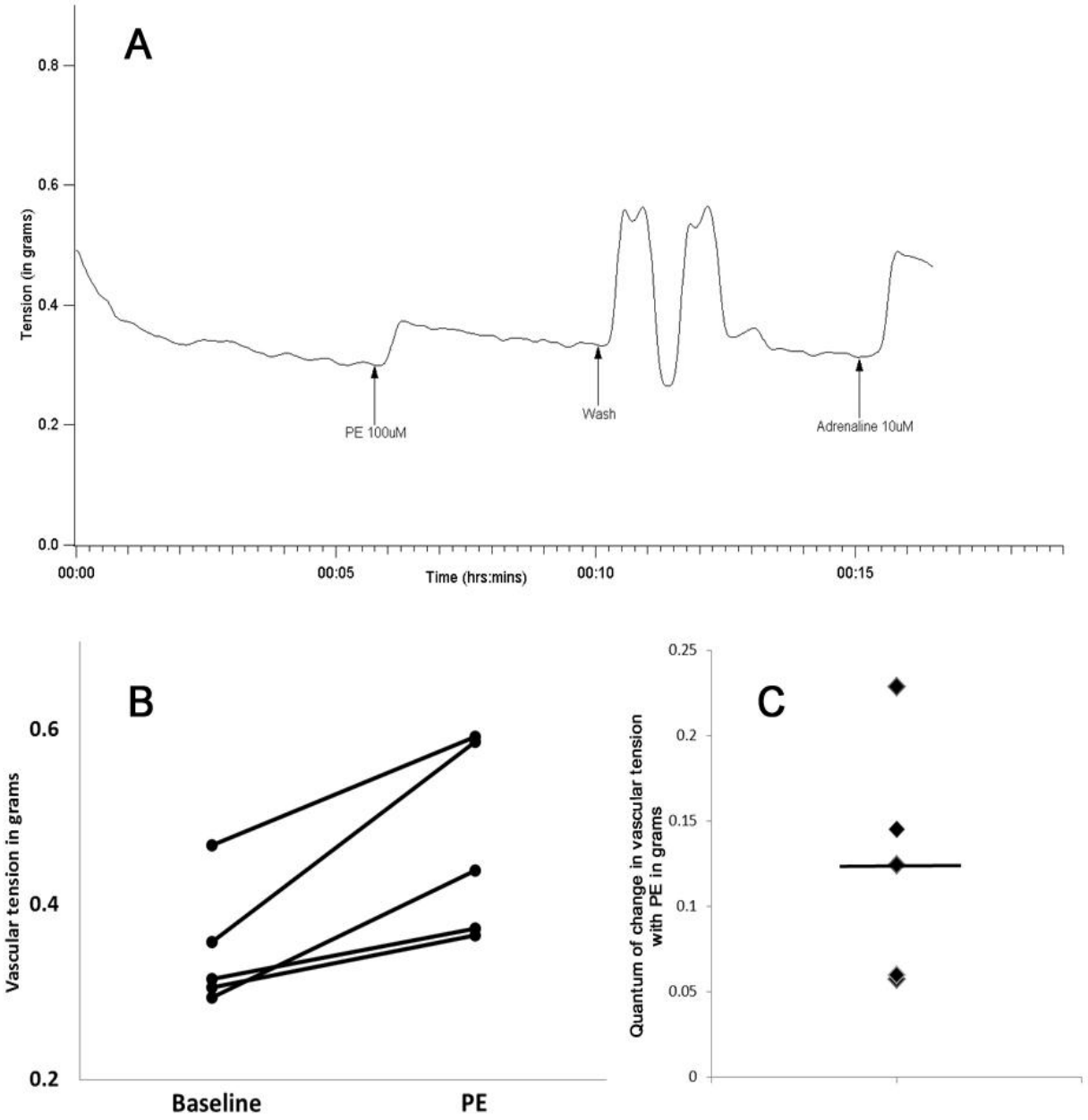


Fig.12

Fig.12: (A) Raw representative tracing showing an increase in vascular tension with PE in longitudinal aortic preparation. (B) Scatter plots of results from all five experiments demonstrating increase in vascular tension with PE. (C) Quantum of change in vascular tension with PE from baseline (* $p < 0.05$)

Phenylephrine increased vascular tension in longitudinal aortic preparations even under high NO environment

Intervention 1: L-Arginine followed by PE on longitudinal aortic strips

400µM L-Arginine did not induce any tissue response, while subsequent addition of phenylephrine produced an increase in vascular tension.

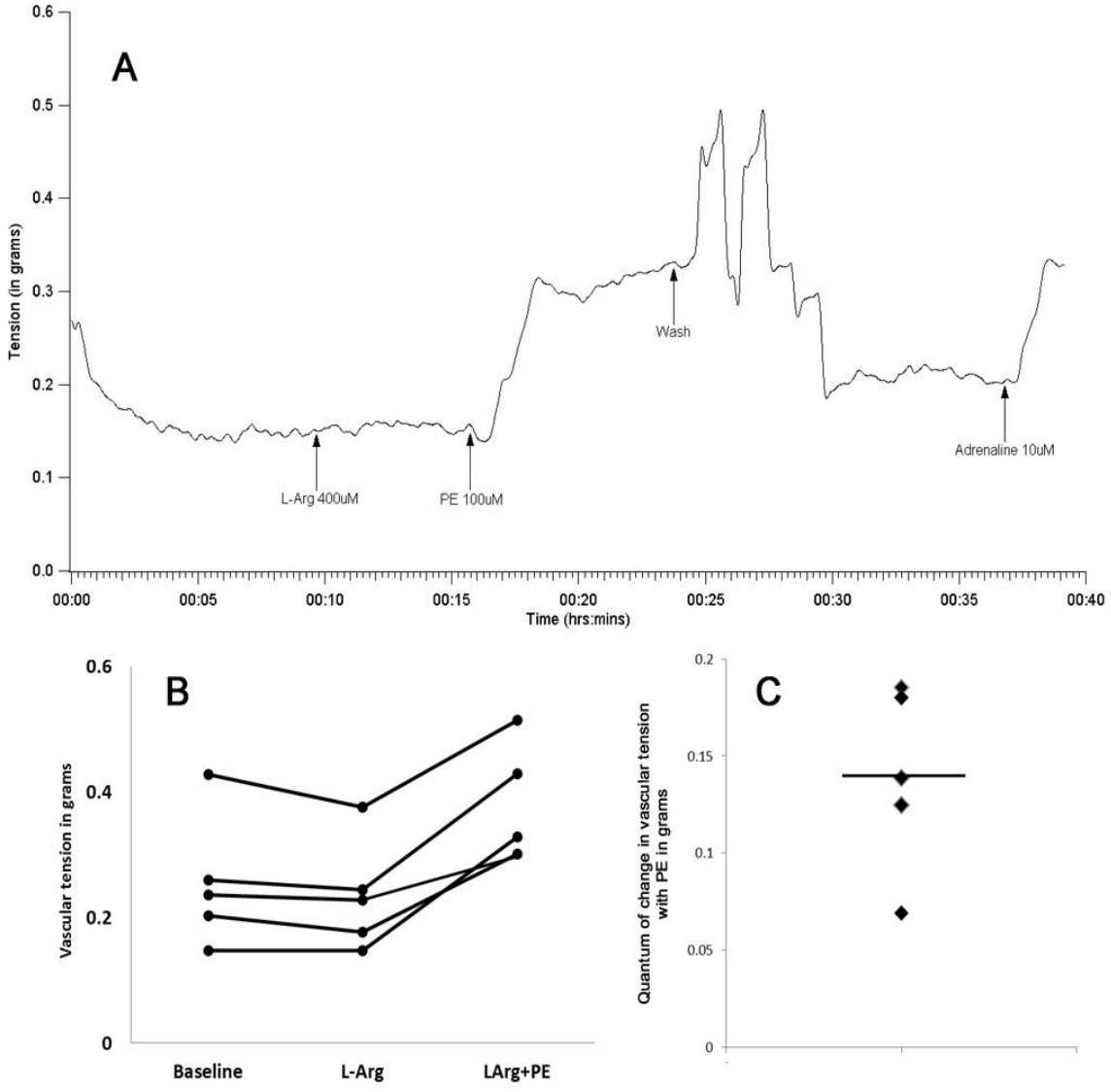


Fig.13

Fig.13: (A) Figure demonstrating the inability of L-Arginine to decrease vascular tension produced by Phenylephrine. (B) Vascular tension profiles due to serial additions of L-Arginine and PE. (C) Quantum of change in vascular tension with PE in the presence of L-Arginine

The vascular tension in the aortic tissue prior to the addition of phenylephrine (in the presence of L-Arginine) was 0.22 gm and the same after addition of phenylephrine was 0.32 gm (median, n=5, p= 0.043* with WSR test). (Fig.13)

There was no significant difference (P = 0.076 with MWU) when the percentage of change in vascular tension due to Phenylephrine with and without L-Arginine were compared, showing that Phenylephrine produced vasoconstriction in aortic longitudinal muscle even under high NO conditions.

Intervention 2: SNP followed by PE on longitudinal aortic strips

400µM sodium nitroprusside did not induce vasorelaxation in the aortic preparation, while subsequent addition of phenylephrine produced an increase in vascular tension.

The vascular tension prior to the addition of PE (in the presence of SNP) was 0.32 g and after addition of PE it was 0.39 g (median, n=5, P= 0.043 with WSR test). (Fig.14)

There was no significant difference (P = 0.251 with MWU) when percentage changes in vascular tension due to PE with and without SNP were compared, indicating no change in the effect of PE alone and when PE is added in presence of NO donors.

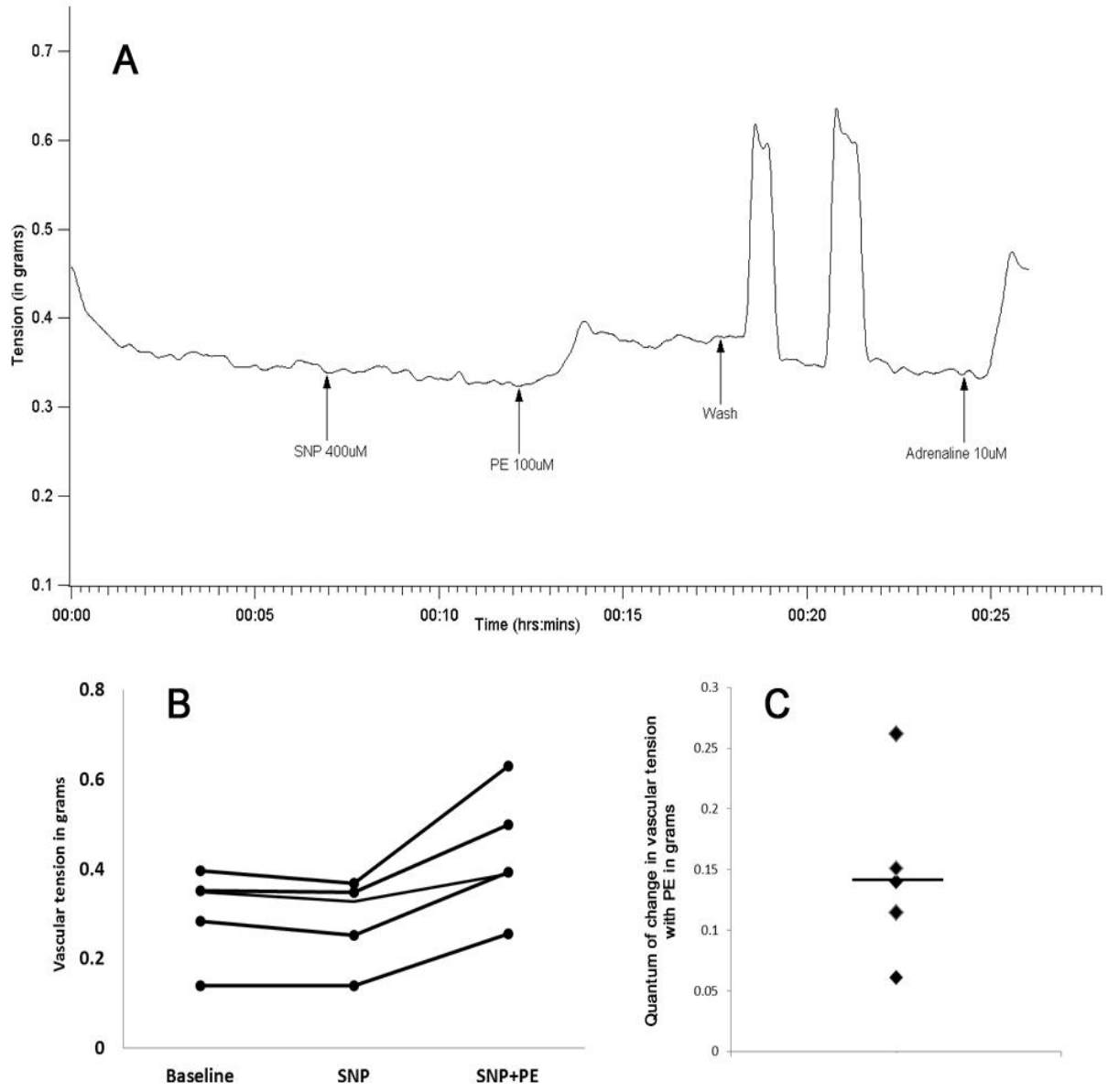


Fig.14

Fig.14: (A) Graphical representation showing the inability of SNP to decrease the vascular tension produced by Phenylephrine (B) Vascular tension profiles due to serial additions of SNP and PE (C) Quantum of change of vascular tension with PE in the presence of SNP.

Phenylephrine produced vasoconstriction in aortic rings under normal conditions

The addition of 100μmol/L phenylephrine significantly increased vascular tension from 0.37 gram to 0.51 gram (median, n = 5) in the transverse cylinders (rings) of aorta. (Fig.15)

Tissue viability was confirmed by vasoconstriction induced by the addition of 10μmol/L adrenaline.

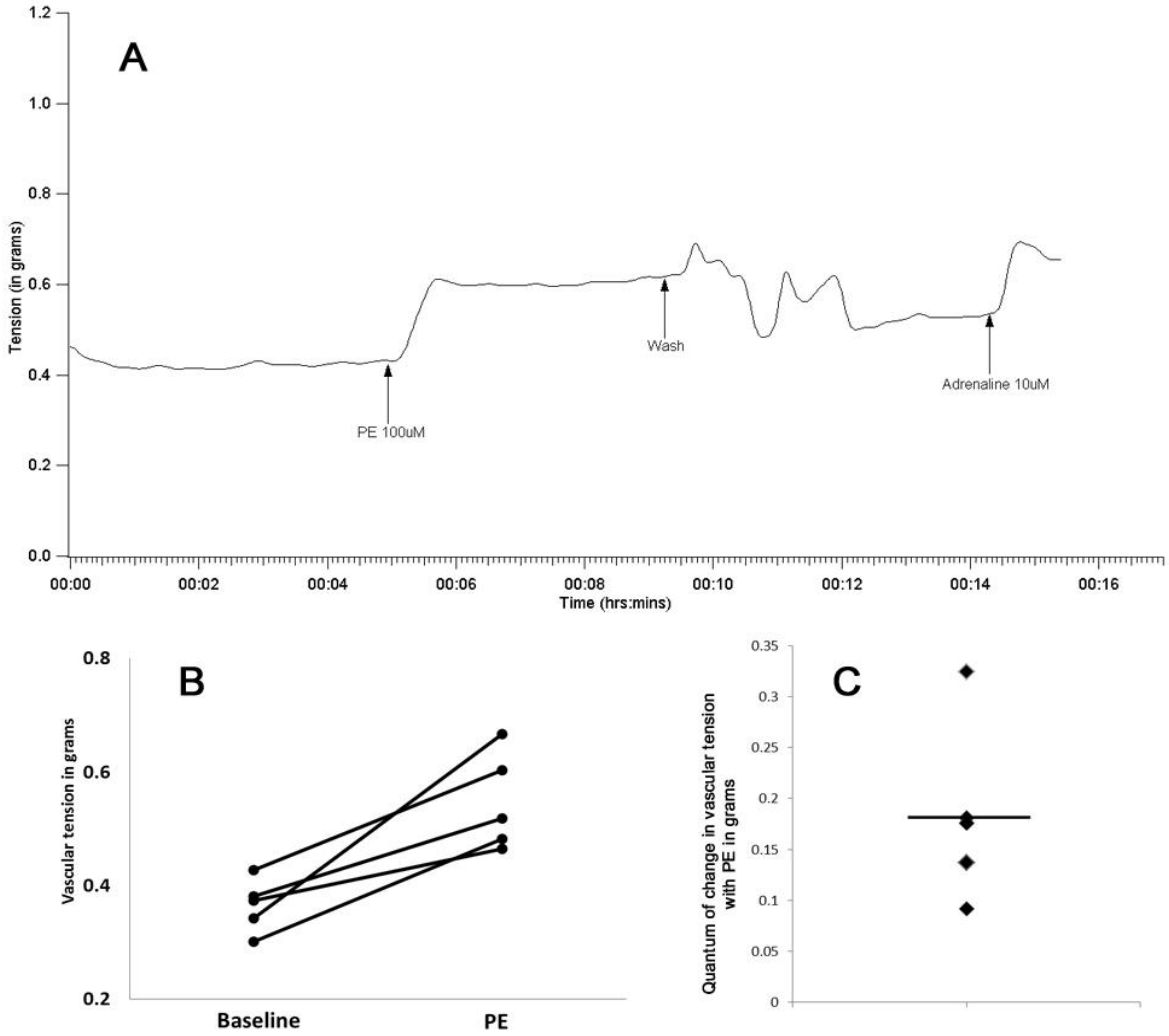


Fig.15

Fig.15: (A) Line tracing showing an increase in vascular tension with PE in aortic ring preparation. (B) Scatter plots of results from all five experiments demonstrating increase in vascular tension with PE. (C) Quantum of change in vascular tension with PE from baseline (* $p < 0.05$)

Phenylephrine increased vascular tension in aortic ring preparations in presence of NO donors

Intervention 1: L-Arginine followed by PE on transverse aortic cylinders (rings)

400µM L-Arginine did not produce apparent vasorelaxation, while subsequent addition of phenylephrine produced an increase in vascular tension.

Before the addition of PE, the aortic vascular tension in the presence of L-Arginine was 0.31 g and after addition of Phenylephrine, it was 0.65 g (median, n=6, P= 0.028 with WSR test). (Fig.16)

There was no significant difference (P = 0.273 with MWU) when percentage change in aortic vascular tension due to Phenylephrine with and without L-Arginine were compared, showing that PE produced vasoconstriction in aortic longitudinal muscle even under high NO conditions.

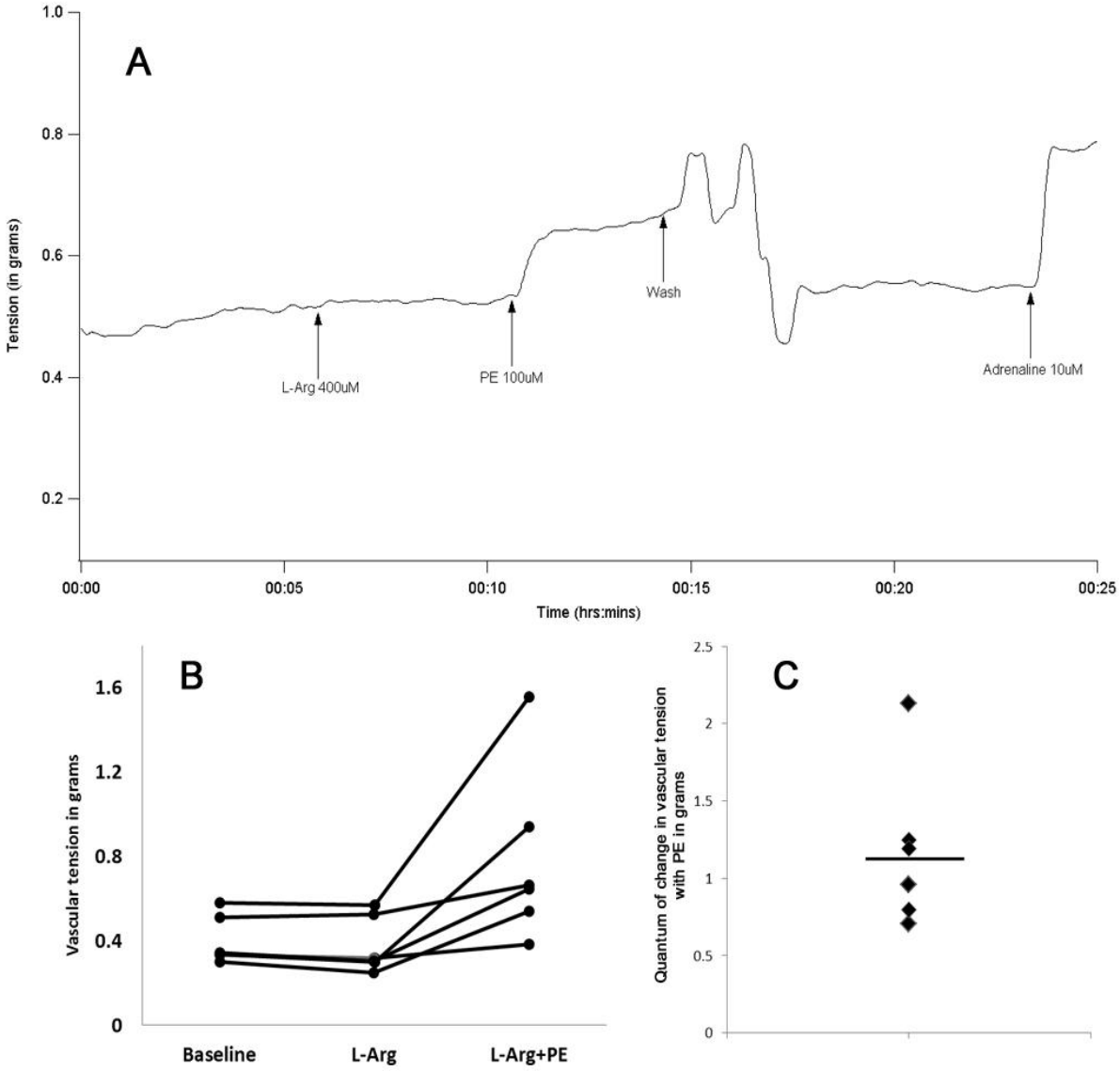


Fig.16

Fig.16: (A) Line tracing demonstrating the inability of L-Arginine to decrease vascular tension produced by Phenylephrine in aortic rings (B) Vascular tension profiles due to serial additions of L-Arginine and PE. (C) Quantum of change in vascular tension with PE in the presence of L-Arginine

Intervention 2: SNP followed by PE on transverse aortic cylinders (rings)

400µM sodium nitroprusside did not induce vasorelaxation, while subsequent addition of phenylephrine produced an increase in vascular tension.

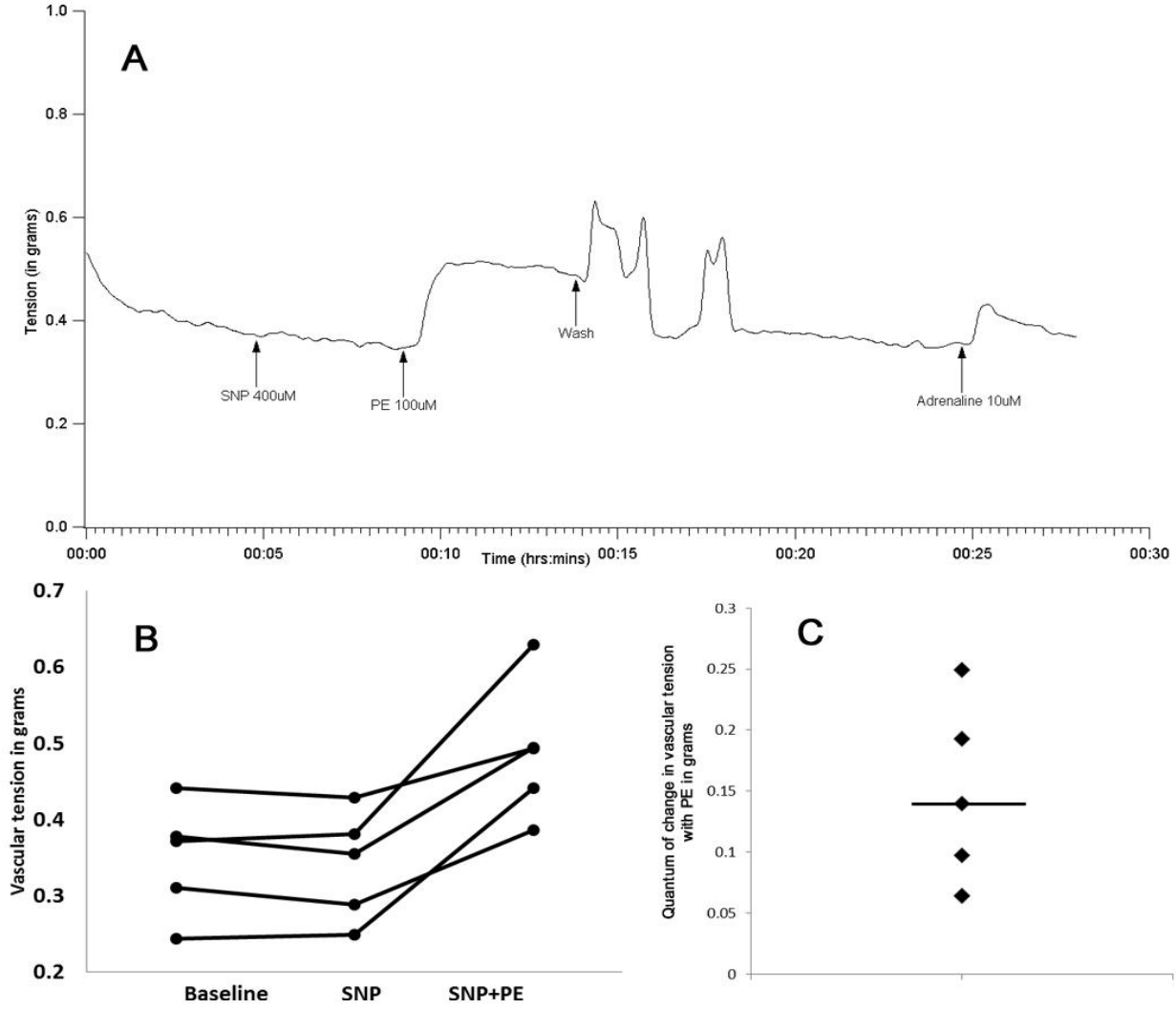


Fig.17

Fig.17: (A) Raw line tracing demonstrating the inability of SNP to decrease vascular tension produced by Phenylephrine. (B) Vascular tension profiles due to serial additions of SNP and PE. (C) Quantum of change in vascular tension with PE in the presence of SNP.

Before the addition of Phenylephrine, in the presence of SNP, the aortic vascular tension was 0.35 g and after addition of PE it was 0.49 g (median, n=5, P= 0.043 with WSR test). (Fig.17)

There was no significant difference (P = 0.754 with MWU) when percentage changes in vascular tension due to PE with and without SNP were compared, indicating no change in the effect of PE in presence of NO donors.

Histology of the experimental tissues

Histology of the tissue samples from the experiments showed that the general architecture of the aorta was maintained and endothelium was intact.

The presence of longitudinal and circular smooth muscles in each of the preparations was confirmed by direct visualization of the nuclear arrangement under microscopy with H&E staining.

In longitudinal sections, the longitudinal smooth muscle fibre nuclei appeared elongated and spindle-shaped, while the nuclei of the circular smooth muscles appeared short and round.

In circular (ring) sections, the circular smooth muscle fibre nuclei appeared elongated and spindle-shaped, while the nuclei of the longitudinal smooth muscles appeared short and round.

Thus, the presence of both lamellar arrangements of vascular smooth muscle in aorta was confirmed and they were found to be intact.

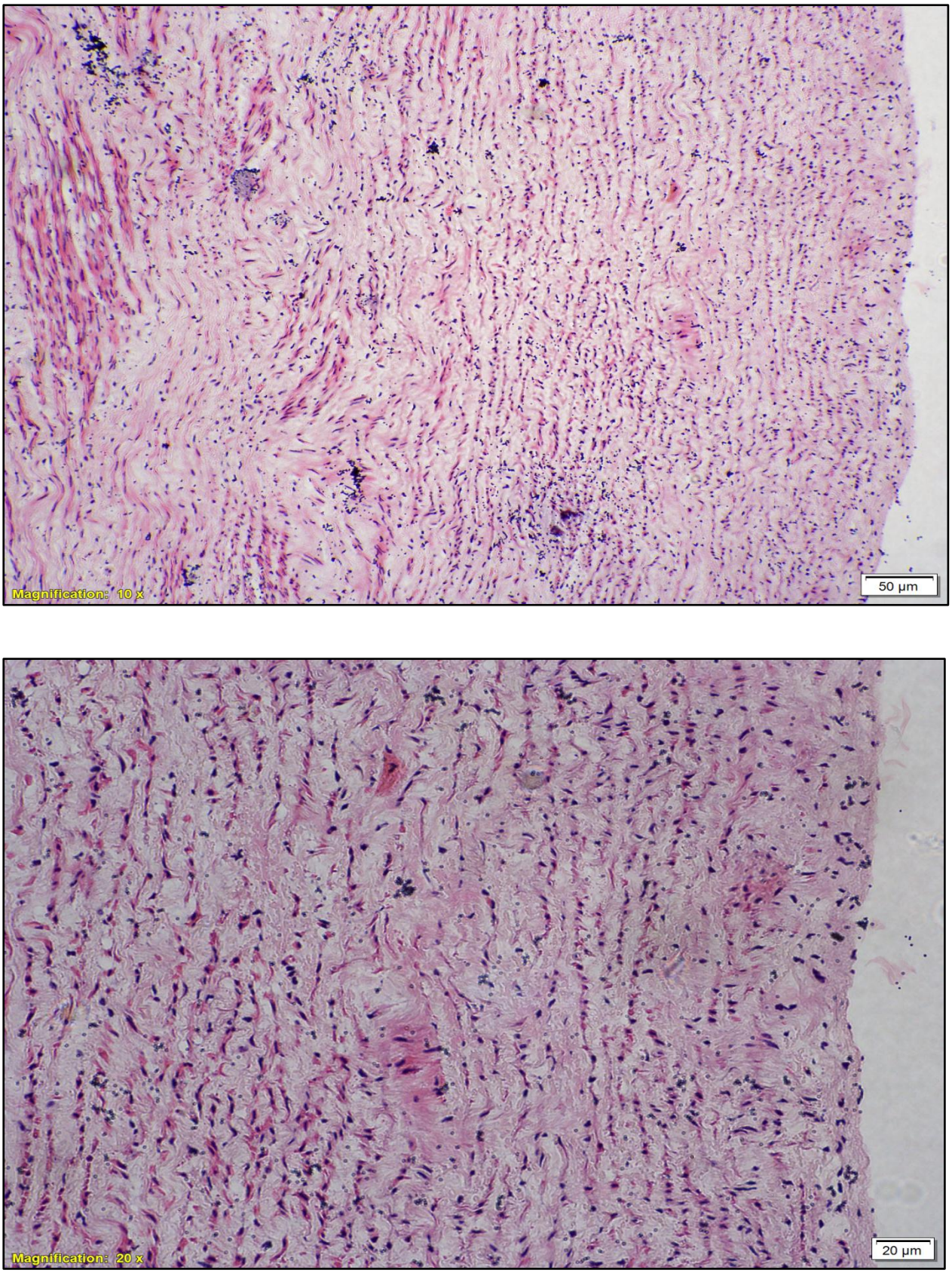


Fig.18. Aorta – Longitudinal strip at (a) 10x and (b) 20x with H&E stain

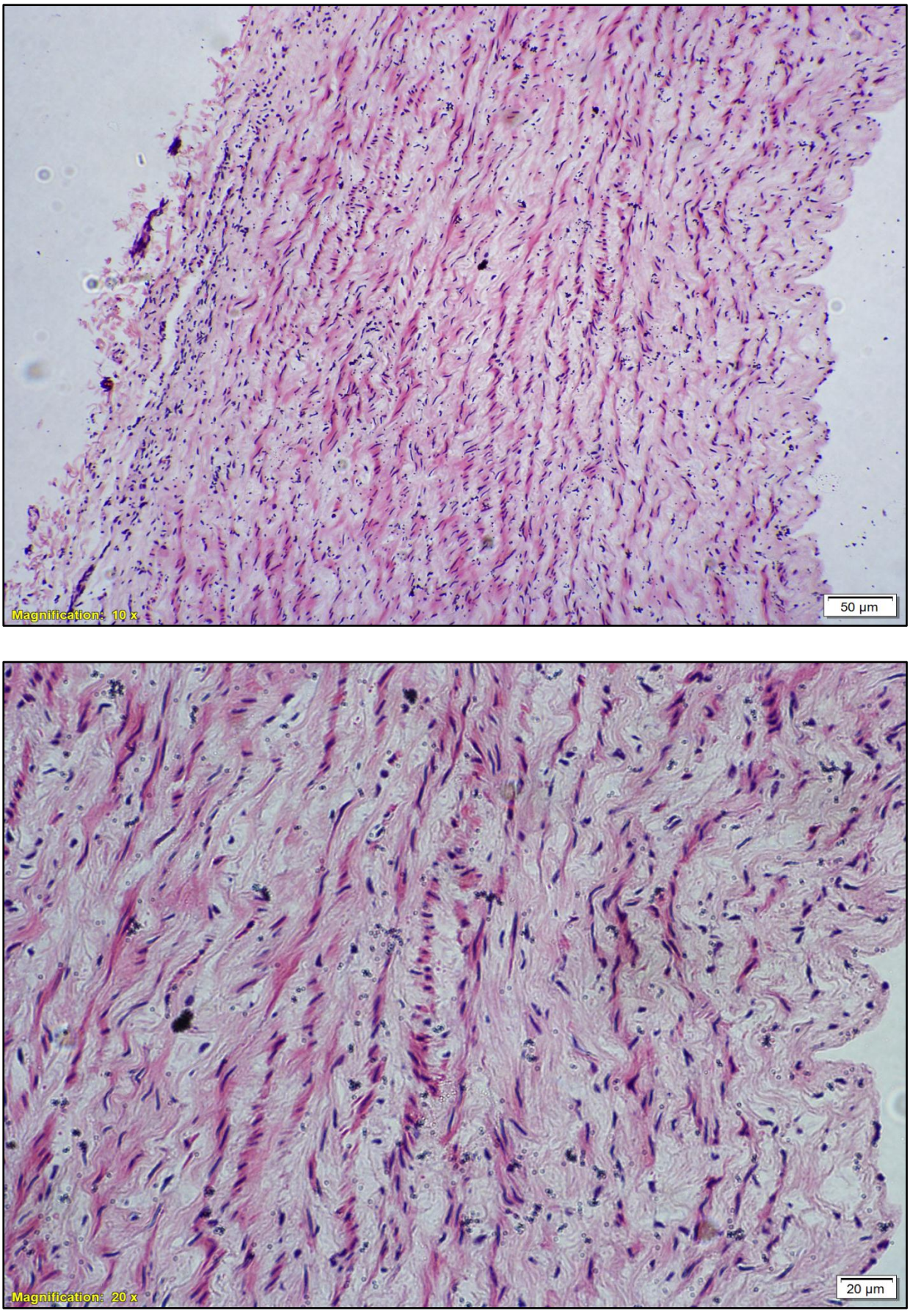


Fig.19. Aorta – Rings at (a) 10x and (b) 20x with H&E stain

DISCUSSION

DISCUSSION

Phenylephrine is a known vasoconstrictor which mediates its effects via the alpha adrenergic receptors and it is widely known as an alpha receptor agonist. It has been used as a vasopressor agent for the clinical management of hypotensive states due to various causes. Earlier findings which have been published from our department show that while phenylephrine did increase vascular tension under control conditions, it led to a fall in vascular tension in spiral strips of peripheral small arteries under certain circumstances where levels of nitric oxide were higher or even at normal levels of nitric oxide, where it is possible that nitric oxide may have been diverted to activate a vasorelaxant pathway (18). Such a vasorelaxant effect produced by phenylephrine is of great concern especially while treating conditions like septic shock, which is a condition where there is excess production of nitric oxide endogenously in the body and medical treatment with alpha agonists like phenylephrine or norepinephrine might further worsen the hypotensive state leading to death. However, it was later demonstrated that the relaxant response was seen only in longitudinal strips and not in transverse preparations. Given this setting, the main aim of this study was to see if the PE-induced vasorelaxation is present in large arteries too. We have also tried to understand how α-adrenergic stimulation of different kinds of aortic smooth muscle responds under normal or high NO conditions.

Here we have assessed whether such a phenylephrine-induced reduction in vascular tone in a high NO environment takes place in a large artery like aorta. In order to delineate its effects on the different arrangements of smooth muscle in the aorta, two different preparations were tested – longitudinal strips for longitudinal muscle action

and transverse cylinders or rings, for the action of circular muscle fibres. While PE alone produced vasoconstriction under normal NO conditions – in line with its popularly known action – the presence of NO donors like L-Arginine and sodium nitroprusside to simulate a high NO environment, did not produce any alteration in the PE-induced vasoconstriction in both aortic preparations. The inference that followed the experiments with L-Arginine and SNP was that vasoconstriction with PE in aorta is not affected under high NO environments.

This finding may help further our knowledge of conditions like septic shock, where there is an excess production of endogenous NO in the body and hence, there is an irreversible vasoplegia of the peripheral small arteries. The widely accepted drugs for medical management in these cases are adrenergic vasopressors that act on alpha adrenoceptors, like phenylephrine, adrenaline and noradrenaline, in a bid to maintain perfusion to vital organs of the body by producing vasoconstriction.

Hence, the results of this study paves way to add to our understanding that the clinical effects of phenylephrine may be different under normal conditions as opposed to conditions where there is an excess of nitric oxide levels in the circulation. In such settings, the action of PE also varies depending on the type of blood vessel it acts on.

CONCLUSION

CONCLUSION

In contrast to earlier findings from our lab in small arteries, there is no indication of any alpha-adrenoceptor mediated vasorelaxant pathway in the aortic smooth muscle.

Further studies are required to correlate with the results obtained from this study and confirm the absence of this mechanism in aorta.

The absence of this pathway in aorta sheds valuable light on the differing actions of phenylephrine on the peripheral small arterioles and central large arteries, under normal conditions and in situations where there is high nitric oxide.

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ANNEXURE

INSTITUTIONAL REVIEW BOARD (IRB) APPROVAL LETTER FOR STUDY

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Anna Benjamin Pulimood, M.B.B.S., MD., Ph.D.,
Chairperson, Research Committee & Principal

Dr. Biju George, M.B.B.S., MD., DM.,
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

April 19, 2017

Dr. Alen Major Venis,
PG Registrar,
Department of Physiology,
Christian Medical College,
Vellore – 632 002.

Sub: **Fluid Research Grant NEW PROPOSAL:**
Delineation of signaling pathway in alpha-adrenoceptor mediated vasorelaxation using goat arterial strips.
Dr. Alen Major Venis, Employment Number: 21338, PG Registrar, Dr. Sathya Subramani, Employment Number: 1412, Head of the Department, Dr. Renu Raj R, Employment Number: 21093, Dr. Bhavithra Bharathi S, Employment Number: 21237, Department of Physiology.

Ref: IRB Min. No. 10412 dated 05.12.2016

Dear Dr. Alen Major Venis,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

CC: Dr. Sathya Subramani, Department of Physiology, CMC

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294. 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

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Ref: IRB Min. No. 10412 dated 05.12.2016

Dear Dr. Alen Major Venis,

The Institutional Review Board **(Blue**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Delineation of signaling pathway in alpha-adrenoceptor mediated vasorelaxation using goat arterial strips" on December 05th 2016.

The Committee reviewed the following documents:

1. IRB Application format
2. Cvs of Drs. Sathya, Alen, Renu, Bhavitha.
3. No. of documents 1 – 2.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294. 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

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Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on December 05th 2016 in the BRTC Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC,Vellore	Internal, Clinician
Dr. B. J. Prashantham	MA(Counseling Psychology), MA (Theology), Dr. Min (Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Ratna Prabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Rekha Pai	BSc, MSc, PhD	Associate Professor, Pathology, CMC, Vellore	Internal,Basic Medical Scientist
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Simon Pavamani	MBBS, MD	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Ms. Grace Rebekha	M.Sc., (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Santhanam Sridhar	MBBS, DCH, DNB	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Delineation of signaling pathway in alpha-adrenoceptor mediated vasorelaxation using goat arterial strips." on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Fluid Grant Allocation:

A sum of 99,500/- INR (Rupees Ninety nine thousand five hundred Only) will be granted for 2 Years.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM.
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min. No. 10412 dated 05.12.2016

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